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(54) Title: **SYNTHETIC INTERNAL RIBOSOME ENTRY SITES AND METHODS OF IDENTIFYING SAME**

(57) Abstract: Synthetic and isolated translational regulatory elements, including oligonucleotides that have translational enhancing activity, internal ribosome entry site (IRES) activity, or translational inhibitory activity, and multimers of such translational regulatory elements are provided. In addition, compositions that include such translational regulatory elements are provided, as are methods of using the translational regulatory elements.

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## SYNTHETIC INTERNAL RIBOSOME ENTRY SITES AND METHODS OF IDENTIFYING SAME

This application claims the benefit of priority under 35 U.S.C. § 119(e) of  
5 U.S. Serial No. 60/230,956, filed September 7, 2000; U.S. Serial No. 60/230,852,  
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January 28, 2000; and U.S. Serial No. \_\_\_\_\_ (attorney docket SCRIP1370), filed  
January 12, 2001, each of which is incorporated herein by reference.

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certain rights in this invention.

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### **BACKGROUND OF THE INVENTION**

#### **FIELD OF THE INVENTION**

The present invention relates generally to compositions for modulating the level  
of translation of a polypeptide, and more specifically to synthetic internal ribosome entry  
site (IRES) nucleotide sequence and to methods of identifying a synthetic IRES.

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#### **BACKGROUND INFORMATION**

Molecular biology provides tools for genetically engineering organisms to  
express large amounts of useful gene products, including new and useful gene  
products. As such, medically useful gene products such as growth factors, hormones,  
25 and immunoregulatory polypeptides, for example, interferons and interleukins are  
now available in sufficient quantities for therapeutic use. In addition, molecular  
biology has allowed gene therapy to evolve to a form that it promises to provide new  
ways for doctors to manage previously untreatable conditions.

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Although molecular biology currently provides tools for expressing  
recombinant proteins in the cells of bacteria, plants, and animals, including human  
cells, methods for manipulating the amount of a protein that is expressed in the cells

are limited. For example, the recombinant protein can be expressed from a particular gene promoter, that has a known level of activity or that is expressed only in one or a few cell types. However, while, the use of a particular promoter may result in the expression of a particular amount of RNA encoding the protein, the amount of protein translated from the RNA does not always correlate directly with the amount of RNA that is expressed. For example, some of the RNA may be degraded in the particular cell, or the RNA may not be translated efficiently. In other cases, the protein may be translated in an amount that is toxic, either to the cell expressing the protein or to another cell that is in proximity to a cell that produces and secretes the protein.

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Nucleotide sequences that are involved in regulating the translation of a polypeptide have been described, and can be utilized to help regulate the amount of a protein translated from an RNA molecule. Some of these nucleotide sequences are functional only when positioned at the 5' end of an RNA molecule, and are useful for regulating the expression of a protein encoded by the nucleotide immediately downstream of the translation regulatory element. However, such nucleotide sequences are not useful for regulating the expression of a number of proteins that are encoded by a single RNA molecule containing a series of open reading frames. For this purpose, nucleotide sequences that can regulate translation from within a nucleotide sequence can be useful. However, only a few such sequences, referred to as internal ribosome binding sites (IRES), have been described, and they generally are very large, containing more than a hundred, generally several hundred, nucleotides.

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The large size of the known IRES sequences limits their usefulness. For example, many gene therapy vectors such as retrovirus vectors are limited in the size of a polynucleotide insert that can be contained in and expressed from the vector. As such, polynucleotides that encode very large proteins cannot be used with certain vectors. The use of an IRES, which can consist of several hundred nucleotides, only further limits the size of the encoding polynucleotide that can be included in the vector, thus further limiting the polynucleotides that can be used in a gene therapy procedure. Furthermore, the use of a single IRES may not result in sufficient expression of a desired polypeptide. Thus, a need exists for regulatory elements that

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are useful for modulating the level of protein expression. The present invention satisfies this need and provides additional advantages.

### **SUMMARY OF THE INVENTION**

5           The present invention relates to an isolated synthetic translational regulatory element, which includes at least one oligonucleotide consisting of about 6 to 125 ribonucleotides, or a deoxyribonucleotide sequence encoding the oligonucleotide. The oligonucleotide is characterized, in part, in having translational regulatory activity, for example, translational enhancing activity, translational inhibitory activity, internal  
10   ribosome entry site (IRES) activity or a combination thereof, and has such translational regulatory activity in a eukaryotic cell.

          A synthetic translational regulatory element of the invention is exemplified herein by an oligonucleotide encoded by any of SEQ ID NOS: 42 to 46, 49, 50, 52, and  
15   89 to 160, which were identified by screening libraries of randomized oligonucleotides. In addition, a synthetic translational regulatory element is exemplified herein by an oligonucleotide that is complementary to an oligonucleotide sequence of a ribosomal RNA, particularly to an oligonucleotide sequence of an un-base paired region of ribosomal RNA, for example, an oligonucleotide encoded by any of SEQ ID NOS: 2,  
20   30, 32, 34, 36, 38, 40 and 50.

          A synthetic translational regulatory element of the invention can contain two or more oligonucleotides having translational regulatory activity, wherein the oligonucleotides are operatively linked to each other, and can be the same or different.  
25   Examples of such synthetic translational regulatory elements include those encoded by a nucleotide sequence selected from any of SEQ ID NOS: 42 to 46, 49, 50, 52, and 89 to 160. In one embodiment, a synthetic translational regulatory element contains five operatively linked oligonucleotides having translational regulatory activity. In another embodiment, a synthetic translational regulatory element contains ten operatively linked  
30   oligonucleotides having translational regulatory activity. In still another embodiment, a synthetic translational regulatory element contains up to fifty operatively linked oligonucleotides having translational regulatory activity. Such oligonucleotides in a



synthetic translational regulatory element can be adjacent to each other, or can be separated from each other by a spacer nucleotide sequence, which can consist of about 1 to 100 ribonucleotides, for example, the synthetic translational regulatory elements encoded by any of SEQ ID NOS: 53 to 88.

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The present invention also relates to a vector containing a synthetic translational regulatory element of the invention. In one embodiment, the vector is an expression vector, which can contain a translation initiation site in addition to the synthetic translational regulatory element, which can have, for example, translational enhancing activity. In addition, the vector can contain a translation start codon operatively linked to the translation initiation site. In another embodiment, the expression vector contains an expressible polynucleotide, wherein the translational regulatory element is operatively linked to the expressible polynucleotide, and wherein the translational regulatory element has transcriptional enhancing activity or IRES activity. The expressible polynucleotide can include one cistron or can be polycistronic, for example, dicistronic. Also provided is a host cell containing a synthetic translational regulatory element of the invention.

The present invention also relates to an isolated translational regulatory element, which includes at least one 5' untranslated region (5' UTR) of a eukaryotic messenger RNA (mRNA) or an oligonucleotide portion thereof, or a deoxyribonucleotide sequence encoding said 5' UTR or oligonucleotide portion thereof. The 5' UTR or oligonucleotide portion thereof is characterized, in part, in having translational enhancing activity, internal ribosome entry site (IRES) activity or a combination thereof, and the translational regulatory activity is effective in a eukaryotic cell.

In one embodiment, the 5' UTR of an isolated translational regulatory element is encoded by a nucleotide sequence selected from any of SEQ ID NOS: 1, 23 to 29, 161, 162, and 164, and the 5' UTR or oligonucleotide portion thereof has translational enhancing activity or IRES activity or both. An oligonucleotide portion of a 5' UTR in such an isolated translational regulatory element is exemplified by an oligonucleotide encoded by any of nucleotides 1 to 40, 1 to 81, 1 to 120, 41 to 81, 14 to 196, 80 to 120,

80 to 196, 120, to 166, and 120 to 196 of a Gtx homeodomain 5' UTR (SEQ ID NO: 1). A particular oligonucleotide portion of the Gtx 5' UTR is shown as SEQ ID NO: 2, which can be linked at its upstream (5') end or downstream (3') end or both ends to a spacer nucleotide sequence, which can be about 1 to 100 ribonucleotides in length, for example, an oligonucleotide encoded by SEQ ID NOS: 4, 5, 12, and 20 to 22. An isolated translational regulatory element also can contain two or more oligonucleotide portions encoded by SEQ ID NO: 2, which are operatively linked to each other, for example, five or ten of the oligonucleotides in operative linkage, and each of the oligonucleotides (SEQ ID NO: 2) can, but need not, be separated from each other by a spacer nucleotide sequence. Examples of such isolated translational regulatory elements are encoded by any of SEQ ID NOS: 6 to 11 and 13 to 15.

Additional examples of an oligonucleotide portion of a 5' UTR having translational regulatory activity including oligonucleotide portions of the yeast YAP1 mRNA (SEQ ID NOS: 30 and 32), and oligonucleotide portions of the yeast p150 mRNA, including any of nucleotides 1 to 250, 100 to 508, 160 to 508, 250 to 508, 375 to 508, 429 to 508, 481 to 508, and 250 to 390 of SEQ ID NO: 29; or any of SEQ ID NOS: 36, 38 and 40. In addition, oligonucleotide sequences of the mouse Rbm3 mRNA, including any of SEQ ID NOS: 165 to 169, 171 to 176, and 182 to 192 have translational regulatory activity. An isolated translational regulatory element also can include at least two 5' UTRs or oligonucleotide portions thereof, wherein the at least two 5' UTRs or oligonucleotide portions are operatively linked to each other, and wherein each of the at least two 5' UTRs or oligonucleotide portions independently is the same or different from each other.

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In another embodiment, an isolated translational regulatory element includes an oligonucleotide portion of a 5' UTR encoded by any of SEQ ID NOS: 1, 23 to 29, 161, 162, and 164, wherein said oligonucleotide portion thereof has translational inhibitory activity. Examples of such oligonucleotides having translational inhibitory activity include those encoded by nucleotides 120 to 196 and 167 to 196 of the Gtx homeodomain protein mRNA (SEQ ID NO: 1), those encoded by nucleotides 1 to 100 and 100 to 160 of the yeast p150 mRNA (SEQ ID NO: 29), the portion of the yeast p150

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mRNA shown as SEQ ID NO: 34, and those encoded by SEQ ID NO: 193 and SEQ ID NO: 194, which encode portions of the mouse Rbm3 mRNA.

The present invention also relates to a kit, which contains a synthetic  
5 translational regulatory element or an isolated translational regulatory element of the invention. In particular, a kit of the invention can contain a plurality of synthetic transcriptional regulatory elements, which are the same as or different from each other, and which can contain a flanking sequence independently at a 5' end or a 3' end or both 5' and 3' ends, to facilitate operatively linking two or more synthetic translational  
10 regulatory elements in the plurality to each other.

The present invention further relates to a recombinant nucleic acid molecule, which contains a synthetic translational regulatory element operatively linked to an expressible polynucleotide. The expressible polynucleotide can comprise a cistron, and  
15 the synthetic translational regulatory element has translational enhancing activity or IRES activity. The expressible polynucleotide also can comprise, in operative linkage in a 5' to 3' orientation, a first cistron, a spacer nucleotide sequence, and a second cistron, and the synthetic translational regulatory element can have IRES activity, and be operatively linked to the second cistron. The synthetic translational regulatory element  
20 in a recombinant nucleic acid molecule of the invention can include two or more operatively linked oligonucleotides having translational regulatory activity.

The polypeptide encoded by a recombinant nucleic acid molecule of the invention can be any polypeptide. A polypeptide encoded by a recombinant nucleic  
25 acid molecule can be any other polypeptide, for example, a viral or bacterial polypeptide or an epitope expressed by a virus, bacterium or other pathogenic organism; or a growth factor, a hormone and a receptor for a growth factor or a hormone. The recombinant nucleic acid molecule also can encode two or more polypeptides.

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A particularly useful polypeptide that can be expressed by a recombinant nucleic acid molecule of the invention, or that can be encoded by an expressible

polynucleotide useful in a composition or method of the invention is a reporter molecule, which provides a means to detect, select or isolate, for example, a cell expressing the reporter polypeptide. Reporter molecules (polypeptides) are well known in the art and include, for example, fluorescent polypeptides such as green fluorescent protein, cyan fluorescent protein, red fluorescent protein, or enhanced forms thereof, an antibiotic resistance polypeptide such as puromycin N-acetyltransferase, hygromycin B phosphotransferase, neomycin (aminoglycoside) phosphotransferase, and the Sh ble gene product, a cell surface protein marker such as the cell surface protein marker is neural cell adhesion molecule (N-CAM), an enzyme such as  $\beta$ -galactosidase, chloramphenicol acetyltransferase, luciferase, and alkaline phosphatase, or a peptide tag such as a c-myc peptide, a polyhistidine, or the like.

Expression of a reporter molecule can be detected using the appropriate reagent, for example, by detecting fluorescence of a green fluorescent protein or light emission upon addition of luciferin to a luciferase reporter molecule, or by detecting binding of nickel ion to a polypeptide containing a polyhistidine tag. Furthermore, the reporter molecule can provide a means of isolating the expressed reporter molecule or a cell expressing the reporter molecule. For example, where the reporter molecule is a polypeptide that is expressed on a cell surface and that contains an operatively linked c-myc epitope, an anti-c-myc epitope antibody can be immobilized on a solid matrix and cells, some of which express the tagged polypeptide, can be contacted with the matrix under conditions that allow selective binding of the antibody to the epitope. Unbound cells can be removed by washing the matrix, and bound cells, which express the reporter molecule, can be eluted and collected. Methods for detecting such reporter molecules and for isolating the molecules, or cells expressing the molecules, are well known to those in the art (see, for example, Hopp et al., BioTechnology 6:1204, 1988; U.S. Pat. No. 5,011,912; each of which is incorporated herein by reference).

The present invention also relates to a method of producing a genetically modified cell that exhibits altered expression of a polypeptide. The method can be performed, for example, by introducing a synthetic translational regulatory element of

the invention into a cell, whereby the synthetic translational regulatory element operatively linked to a nucleotide sequence encoding a polypeptide, thereby producing a genetically modified cell that exhibits altered expression of a polypeptide. In one embodiment, the synthetic translational regulatory element is operatively  
5 linked to the nucleotide sequence prior to introducing the synthetic translational regulatory element into the cell, wherein the synthetic translational regulatory element has translational enhancing activity or IRES activity. The polypeptide can be any polypeptide, for example, a reporter polypeptide, is a toxin, or a therapeutic agent. The synthetic translational regulatory element can be stably maintained in the cell, for  
10 example, by integration into the cell genome.

In another embodiment, the nucleotide sequence to which the synthetic translational regulatory element is operatively linked is a sequence of an endogenous gene in the cell genome. The synthetic translational regulatory element can have  
15 translational inhibitory activity, translational enhancing activity or IRES activity. Accordingly, the present invention also relates to a genetically modified eukaryotic cell produced by a method of the invention, as well as to a transgenic non-human eukaryotic organism containing or derived from such a genetically modified cell, a cell or tissue obtained from the transgenic non-human eukaryotic organism, and a  
20 cDNA or genomic DNA library prepared from the transgenic non-human eukaryotic organism or from a cell or tissue obtained from said transgenic non-human eukaryotic organism.

The present invention further provides a method of altering translational activity  
25 in a eukaryotic cell, the method comprising introducing into the cell a synthetic translational regulatory element, whereby the synthetic translational regulatory element interacts with a translation regulatory factor in the cell, thereby altering translational activity in the eukaryotic cell. In addition, the invention provides a method of altering translational activity in a eukaryotic cell, the method comprising introducing into the cell  
30 an isolated translational regulatory element, whereby the synthetic translational regulatory element interacts with a translation regulatory factor in the cell, thereby altering translational activity in the eukaryotic cell. The translational regulatory element

can have translational enhancing activity or IRES activity, whereby translational activity in the cell is decreased, or can have translational inhibitory activity, whereby translational activity in the cell is decreased.

5           The present invention also provides a method of improving protein yield by a eukaryotic cell. Such a method can be performed, for example, by introducing into the cell a recombinant nucleic acid molecule containing a translational regulatory element operatively linked to an expressible polynucleotide, and expressing a polypeptide encoded by the expressible polynucleotide in the cell. The translational  
10 regulatory element generally has translational enhancing activity or IRES activity. In one embodiment, the expressible polynucleotide comprises a first cistron encoding a polypeptide that enhances protein stability or cell viability, and can also comprises a second cistron, which can encode any polypeptide of interest. Where the expressible polynucleotide contains two, or more, cistrons, the second, and other, cistron is  
15 operatively linked to the first cistron. Preferably, such an expressible polynucleotide contains one or more IRES elements, which can be operatively linked to the first cistron or the second or other cistrons or to all of the cistrons, and can be operatively linked to each other to form a concatemer of IRES elements. In such a method, the polypeptide that enhances protein stability or cell viability can be any polypeptide, for  
20 example, a chaperone protein, a heat shock protein, a protein having anti-oxidant activity, a protease inhibitor, a phosphatase inhibitor, a caspase inhibitor, or an antibiotic peptide such as a magainin, a defensin, or a cryptdin.

          In another embodiment, the expressible polynucleotide comprises a first  
25 cistron encoding a polypeptide that enhances transcription or translation of a polynucleotide in the cell, for example, a transcription factor or a translation regulatory factor such as eukaryotic initiation factor or ribosomal protein or the like. The expressible polynucleotide also can comprise a second operatively linked cistron encoding a polypeptide of interest, which can be under the control of an IRES element  
30 of the invention, and the polypeptide that enhances transcription or translation of a polynucleotide can be a polypeptide that enhances transcription or translation of the expressible polynucleotide.

The present invention further relates to a method of expressing a polypeptide in a eukaryotic cell. Such a method can be performed, for example, by introducing into the cell a recombinant nucleic acid molecule comprising a translational  
5 regulatory element operatively linked to an expressible polynucleotide, wherein the translational regulatory element has an activity selected from translational enhancing activity and IRES activity, and expressing the expressible polynucleotide in the cell. The expressible polynucleotide can comprise a first cistron encoding a therapeutic polypeptide or a reporter polypeptide, which can be useful as a diagnostic agent. A  
10 therapeutic polypeptide can be, for example, an immunomodulator such as a cytokine, a neuromodulator, a hormone such as preproinsulin, a growth factor such as epidermal growth factor, a growth factor receptor such as a PDGF receptor, an apoptotic polypeptide such as Bax, an anti-apoptotic polypeptide such as Bcl-2, or an antibiotic. The expressible polynucleotide also can contain a second cistron encoding a  
15 polypeptide that facilitates expression of the therapeutic or diagnostic polypeptide or that facilitates activity of the therapeutic polypeptide. A polypeptide that facilitates expression or activity of such a polypeptide can be, for example, a transcription factor, which can increase transcription of the expressible polynucleotide; a translational regulatory factor, which can increase translation of the encoded  
20 polypeptide; a chaperone protein, a protein having anti-oxidant activity, a protease inhibitor, or a phosphatase inhibitor.

According to a method of the invention, the expressible polynucleotide can be introduced into a cell *ex vivo*, for example, into a cell in culture, or can be introduced  
25 into a cell *in vivo* in a eukaryotic organism, including a mammalian organism such as a human. As such, the recombinant nucleic acid molecule can be contained in a vector, which can be a plasmid vector or a viral vector such as an adenovirus vector, an adeno-associated virus vector, or a retrovirus vector. Such a method can be useful for treating or diagnosing a pathologic condition, or to monitor the progression of a  
30 pathologic condition.

The present invention also relates to a method of identifying a cell. Such a method can be performed by introducing into the cell a recombinant nucleic acid molecule comprising a translational regulatory element of the invention operatively linked to an expressible polynucleotide, wherein the translational regulatory element has an activity selected from translational enhancing activity and IRES activity, wherein the expressible polynucleotide comprises at least one cistron, which encodes a first reporter polypeptide, and wherein expression of the reporter polypeptide in the cell provides a means to identify the cell. If desired, the expressible polynucleotide also can contain a second cistron encoding a polypeptide of interest, in which case the second cistron is operatively linked to the first cistron, and the expressible polynucleotide also contains a synthetic IRES element of the invention, which can be operatively linked to the first cistron or the second cistron or both. The polypeptide of interest can be any polypeptide, including a second reporter polypeptide, which can be the same as or different from the first reporter polypeptide.

A method of identifying a cell as disclosed herein can further include a step of isolating a cell expressing a reporter polypeptide. In one embodiment, the step of isolating utilizes a reporter polypeptide expressed from the expressible polynucleotide. Thus, the reporter polypeptide can be, for example, a cell surface marker, wherein isolating the cell expressing the cell surface marker utilizes an antibody that specifically binds the cell surface marker. A cell surface marker can be a polypeptide that is normally expressed on a cell surface, for example, N-CAM, and the antibody can be an anti-N-CAM antibody, or the cell surface marker can comprise a peptide tag, which is expressed as part of a cell surface protein, in which case the cell can be isolated using an antibody specific for the cell surface protein, or an antibody or other ligand specific for the peptide tag. Accordingly, the present invention also provides an isolated cell obtained according to a method of the invention.



### **BRIEF DESCRIPTION OF THE FIGURES**

Figure 1 illustrates the structure of Gtx (SEQ ID NO: 1) deletion constructs used to identify sequences having translational regulatory activity, and shows the relative IRES activity as determined using the RPh construct (see Example 1). Gtx1-196/RPh indicates a portion of the RPh plasmid containing a nucleotide sequence encoding the 196 nucleotide Gtx 5' untranslated region (5' UTR). Nucleotides included in the various constructs are indicated. Relative IRES activity is shown as a percent of the activity of the full length Gtx 5' UTR. Numbers in parentheses indicate standard error.

Figure 2 shows a portion of the ribonucleotide sequences of various Gtx IRES module (SEQ ID NO: 2) constructs. SEQ ID NOS: of encoding deoxyribonucleotide sequences are indicated in parentheses. Nucleotide sequences as shown correspond, for example, to nucleotides 1 to 27 of SEQ ID NO: 6 (5 copies of wild-type Gtx IRES), SEQ ID NOS: 17-19, containing 1, 2 or 3 substitutions at the 3' end of each Gtx IRES module in the repeated unit, and SEQ ID NOS: 20-22, containing 1, 2 or 3 substitutions at the 5' end of the spacer sequence in each repeated unit. Relative IRES activity as a ratio of *Renilla* luciferase activity to *Photinus* luciferase activity (R:P) is shown, normalized to R:P activity of the RPh vector, alone.

Figure 3 shows regions of complementarity between sequences of yeast 18S ribosomal RNA (SEQ ID NOS: 31, 33, 35, 37 and 39) and oligonucleotide portions of the yeast YAP1 5' UTR (SEQ ID NOS: 30 and 32) or the yeast p150 5' UTR (SEQ ID NOS: 34, 36, 38 and 40). Nucleotide sequences of the yeast YAP1 5' UTR (SEQ ID NO: 28), yeast p150 5' UTR (SEQ ID NO: 29), and 18S ribosomal RNA are indicated.

Figure 4 is a map of the MESVR/EGFP/ECFP/RSVPro(ori) vector (SEQ ID NO: 16) showing the positions of unique restriction endonuclease recognition sites.

Figure 5 shows a region of complementarity of 18S ribosomal RNA (SEQ ID NO: 50) and a synthetic IRES element, ICS1-23 (SEQ ID NO: 49). "a" and "b" indicates portions of SEQ ID NO: 49 that were examined separately for IRES activity. SEQ ID NOS: are indicated in parentheses.

Figure 6 shows deletion constructs of a nucleotide sequence (SEQ ID NO: 164) encoding the mouse Rbm3 5' UTR, and illustrates a portion of the RPh vector in which the sequences were examined for IRES activity. Nucleotide positions relative to the 5' UTR (SEQ ID NO: 164) are indicated in parentheses to the left, and SEQ ID NOS: are indicated in parentheses to the right of the figure. Adenosine residues were added to the 3' deleted sequences (SEQ ID NOS: 181-190) such that the relative distance to the natural Rbm3 AUG start codon was maintained (indicated in Figure as, for example, "(A)40"; see SEQ ID NO: 181). Normalized IRES activity of the various constructs is shown (see description of Figure 4. above).

Figures 7A and 7B illustrate sites in which IRES modules of the invention share complementarity to mouse 18S ribosomal RNA (rRNA; SEQ ID NO: 196).

Figure 7A provides a linear representation of the 18S rRNA, the vertical lines below the linear representation are sites at which selected IRES modules share 8 or 9 nucleotides of complementarity with the to 18S rRNA sequence.

Figure 7B shows a secondary structure of the 18S rRNA, and the dark bars indicate the positions of the complementary sequence matches to selected IRES modules of the invention.

### **DETAILED DESCRIPTION OF THE INVENTION**

The present invention provides a synthetic translational regulatory element, which includes at least one oligonucleotide consisting of about 6 to 125 ribonucleotides, or a deoxyribonucleotide sequence encoding the oligonucleotide. The oligonucleotide is characterized, in part, in having translational regulatory activity, for example, translational enhancing activity, translational inhibitory activity, internal ribosome entry site (IRES) activity or a combination thereof, and has such translational regulatory activity in a eukaryotic cell.

As disclosed herein, a synthetic translational regulatory element can be identified by screening libraries of randomized oligonucleotides and identifying those having translational enhancing activity, translational inhibitory activity, IRES activity, or a combination thereof in a eukaryotic cell. In addition, a synthetic translational regulatory element can be designed based on the nucleotide sequence of a ribosomal RNA (rRNA; see SEQ ID NOS: 195-197; GenBank Accession Nos. V01335, X00686, X03205, respectively, each of which is incorporated herein by reference). In particular, oligonucleotides to be examined for translational regulatory activity, including IRES activity, can be designed so as to be complementary to an oligonucleotide sequence of rRNA, preferably an un-base paired oligonucleotide sequence of rRNA, which is not generally part of a double stranded region that forms due to hybridization of self-complementary sequence of the rRNA (see Figure 7B). Furthermore, the a synthetic translational regulatory element can be identified by screening a variegated population of oligonucleotide sequences (see, for example, U.S. Pat. No. 5,837,500), which can be based, for example, on a translational regulatory element of the invention as disclosed herein or on an oligonucleotide sequence complementary to an un-base paired region of a rRNA.

The present invention also provides an isolated translational regulatory element, which includes at least one 5' untranslated region (5' UTR) of a eukaryotic messenger RNA (mRNA) or an oligonucleotide portion thereof, or a deoxyribonucleotide sequence encoding said 5' UTR or oligonucleotide portion thereof. The 5' UTR or oligonucleotide portion thereof is characterized, in part, in having translational enhancing activity, internal ribosome entry site (IRES) activity or a combination thereof, and the translational regulatory activity is effective in a eukaryotic cell.

As used herein, the term "isolated," when used in reference to a translational regulatory element or a nucleotide sequence encoding the element, indicates that the nucleotide sequence is in a form other than the form in which it is found in nature. Thus, a nucleotide sequence encoding an isolated translational regulatory element is separated, for example, from a gene in which it normally can be found in nature, and particularly from a chromosome in a cell, and an isolated translational regulatory

element is separated, for example, from an mRNA that normally contains the element. It should be recognized, however, that a translational regulatory element of the invention can be linked to additional nucleotide sequences, yet still be considered "isolated" provided the construct comprising the regulatory element is not in a form that is found in nature. Thus, the translational regulatory element can be contained within a vector, or can be operatively linked to a second nucleotide sequence, for example, another regulatory element or an expressible polynucleotide.

The term "synthetic" also is used herein to refer to a translational regulatory element of the invention. For convenience, the term "synthetic" is used herein to refer to a translational regulatory element that is identified by screening a library of oligonucleotides, which can be randomized oligonucleotides, variegated oligonucleotides, or the like. In comparison, the term "isolated translational regulatory element" is used to refer to an element that is identified by examining a naturally occurring nucleotide sequence, particularly a 5' UTR of an mRNA, which can be a 5' UTR of a known mRNA, or can be identified by screening a cDNA library, particularly those portions of the cDNA encoding mRNA 5' UTRs, or by screening a genomic DNA library. Nevertheless, it should be recognized that the means of producing or identifying a translational regulatory element of the invention is not relevant to the composition, activity or use of the element. As such, an isolated translational regulatory element, having been identified from an mRNA, for example, can be produced using routine chemical or biochemical methods of nucleic acid synthesis and, similarly, a library of oligonucleotides used to identify a synthetic regulatory element can contain oligonucleotides that correspond to translational regulatory elements that also can be found in nature.

As disclosed herein, the translational regulatory elements of the invention can have translational enhancing or translational inhibitory activity, and also can have IRES activity, which provides a means to effect cap-independent translation. In eukaryotes, the initiation of mRNA translation is believed to occur generally by a cap-binding/scanning mechanism, although some mRNAs are translated efficiently despite the lack of a cap structure or a free 5' end (Kozak, Gene 234:187-208, 1999;

Gingras et al., Ann. Rev. Biochem. 68:913-963, 1999). In some of these cases, sequences contained within the mRNA nucleotide sequence directly recruit the translation machinery. These sequences, IRESes, are defined functionally using dicistronic mRNAs, wherein a nucleotide sequence is considered to function as an IRES if, when present in the intercistronic region of a dicistronic mRNA, it directs translation of the second cistron in a manner that is independent of the first cistron.

IRESes have been identified in the 5' UTRs of cellular and viral mRNAs, and have been extensively characterized in picornaviral mRNAs, where they comprise well defined segments of about 450 nucleotides (Nicholson et al., J. Virol. 65:5886-5894, 1991). These IRESes have been categorized on the basis of sequence and structural similarities (Jackson and Kaminski, RNA 1:985-1000, 1995). In contrast, the IRES elements of cellular mRNAs are not as well characterized, and in many cases distinct boundaries have been difficult to determine by deletion analysis (Stoneley et al., Oncogene 16:423-428, 1998; Huez et al., Mol. Cell. Biol. 18:6178-6190, 1998; Gan et al., J. Biol. Chem. 273:5006-5012, 1998; Bernstein et al., J. Biol. Chem. 272:9356-9362, 1997, each of which is incorporated herein by reference). However, a 55 nucleotide sequence of the *Drosophila Antennapedia* 5' UTR has been reported to have IRES activity (Oh et al., Genes Devel. 6:1643-1653, 1992, which is incorporated herein by reference).

Cellular IRESes do not contain any obvious sequence similarity to each other or to picornaviral IRES sequences. It has been suggested that IRESes may resemble each other in secondary structure (Le and Maizel, Nucl. Acids Res. 25:362-369, 1997). RNA folding analyses have indicated that some cellular IRESes may contain a Y-type system-loop structure, followed by a stem-loop immediately upstream of the initiation codon. This Y-shaped conformation was predicted to occur in IRES sequences of mRNAs encoding the immunoglobulin heavy chain binding protein (BiP), fibroblast growth factor-2, and the *Antennapedia* gene product, and is reportedly similar to one contained within picornavirus, pestivirus, and hepatitis C virus IRESes (Le et al., Virus Genes 12:135-147, 1996). RNA conformations, including Y-shaped secondary structures, also have been predicted to occur within

several other cellular IRES sequences (Stoneley et al., *supra*, 1998; Huez et al., *supra*, 1998; Sella et al., Mol. Cell. Biol. 19:5429-5440, 1999; Nanbru et al., J. Biol. Chem. 272:32061-32066, 1997). In the c-sis mRNA, for example, three independent fragments have IRES activity, and each fragment is proposed to contain a Y-type stem-loop structure (Sella et al., *supra*, 1999).

No independent physical evidence has demonstrated that the predicted Y-shaped RNA conformations occur in nature, and for many cellular IRESes, the ability to internally initiate translation is not correlated with the presence of the proposed secondary structure. For example, 5' or 3' deletions of the BiP IRES maintain IRES activity even though the deleted fragments do not contain the predicted Y-type stem loop structure (Yan and Sarnow, Nucl. Acids Res. 25:2800-2807, 1997). This lack of correlation between proposed RNA conformations and IRES activity has also been observed in other studies. For example, the vascular endothelial growth factor and c-myc IRESes each contain two non-overlapping fragments with IRES activity (Stoneley et al., *supra*, 1998; Huez et al., *supra*, 1998), and the BiP IRES has three non-overlapping fragments with IRES activity, none of which contain the proposed Y-type stem-loop structure, or, at best, contain only part of it (Yang and Sarnow, *supra*, 1997). Such observations suggest that cellular IRESes may be composed of numerous, short segments that have independent IRES activity or that act together to affect overall IRES activity.

As disclosed herein, analysis of the Gtx 5' UTR (SEQ ID NO: 1) revealed numerous oligonucleotide portions of the 5' UTR having translational regulatory activity (see Example 1). Thus, the present invention provides, for example, an isolated translational regulatory element encoded by any of nucleotides 1 to 40, 1 to 81, 1 to 120, 41 to 81, 14 to 196, 80 to 120, 80 to 196, 120, to 166, and 120 to 196 of the Gtx homeodomain 5' UTR (SEQ ID NO: 1). Even more remarkably, a 9 nucleotide sequence the Gtx homeodomain mRNA 5' UTR (SEQ ID NO: 2; nucleotides 141 of the 196 nucleotide Gtx 5' UTR; SEQ ID NO: 1) was demonstrated to have the characteristics of an IRES element (see Example 1; Komuro et al., EMBO J. 12:1387-1401, 1993, which is incorporated herein by reference). This Gtx IRES module (SEQ

ID NO: 2) functions independently to enhance translational activity, and multiple copies of the IRES module synergistically enhance translational activity (see Example 1). Accordingly, the present invention also provides an isolated translational regulatory element encoded by SEQ ID NO: 2.

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As disclosed herein, the Gtx 5' IRES module (SEQ ID NO: 2) can be linked at its upstream (5') end or downstream (3') end or both ends to a spacer nucleotide sequence, which can be about 1 to 100 ribonucleotides in length such that the activity of the IRES module can be varied. Thus, the invention provides, for example, a translational  
10 regulatory element encoded by any of SEQ ID NOS: 4, 5, 12, and 20 to 22, which includes a Gtx IRES module (SEQ ID NO: 2) and confers variable levels of IRES activity on an operatively linked expressible polynucleotide.

An isolated translational regulatory element also can contain two or more of the  
15 oligonucleotide portions encoded by SEQ ID NO: 2, which are operatively linked to each other, for example, five or ten of the oligonucleotides in operative linkage, and each of the oligonucleotides (SEQ ID NO: 2) can, but need not, be separated from each other by a spacer nucleotide sequence. As disclosed herein, multiple copies of a Gtx IRES module exhibit a greater than additive amount of IRES activity (see Example 1). Thus,  
20 the present invention provides, for example, an isolated translational regulatory elements encoded by any of SEQ ID NOS: 6 to 11 and 13 to 15.

The cooperative increase in IRES activity exhibited by multiple copies of the Gtx IRES module indicates that cellular IRES activity can depend on the strength,  
25 number and spacing of various individual IRES modules. Photochemical crosslinking studies previously demonstrated that this region of the Gtx mRNA sequence binds to 40S ribosomal subunits by crosslinking to its complement within the 18S rRNA (Hu et al., Proc. Natl. Acad. Sci., USA 96:1339-1344, 1999, which is incorporated herein by reference). As disclosed herein, the 9 nucleotide IRES module is 100%  
30 complementary to the 18S rRNA at nucleotides 1132 to 1124.

A further examination of 5' UTRs of eukaryotic mRNAs revealed that translational regulatory activity, including IRES activity, was effected by the 5' UTRs of five dendritically localized mRNAs, including the rat activity related cytoskeletal protein (ARC; see SEQ ID NO: 25), rat dendrin (see SEQ ID NO: 24), rat  
5 microtubule associated protein-2 (MAP2; see SEQ ID NO: 26), the rat  $\alpha$ -Cam Kinase II subunit (CamK IIa; SEQ ID NO: 23), and the rat RC3 neurogranin gene (see SEQ ID NO: 27), by two yeast 5' UTRs, yeast YAP1 (see SEQ ID NO: 28) and yeast p150 (see SEQ ID NO: 29), by the 5' UTRs of mRNAs encoding the rat amyloid precursor protein (APP; see SEQ ID NO: 161), the rat beta-site APP cleaving enzyme  
10 (Bace; SEQ ID NO: 162), the human FMR protein (see SEQ ID NO: 164), and the mouse Rbm3 protein (see SEQ ID NO: 164), each of which internally initiated translation when placed in the intercistronic region of a dicistronic RNA (see Examples 2 and 3). Accordingly, in one embodiment, the present invention provides an isolated translational regulatory element encoded by a nucleotide sequence selected  
15 from any of SEQ ID NOS: 1, 23 to 29, 161 to 164

Detailed analysis of two of the 5' UTRs, yeast p150 and mouse Rbm3, confirmed the results obtained for the Gtx 5' UTR. Thus, oligonucleotide portions of the yeast p150 5' UTR, including, for example, those encoded by any of nucleotides 1 to 250, 100 to  
20 508, 160 to 508, 250 to 508, 375 to 508, 429 to 508, 481 to 508, and 250 to 390 of SEQ ID NO: 29 (see Example 3); and oligonucleotide portions of the mouse Rbm3 5' UTR, including, for example, those encoded by any of SEQ ID NOS: 165 to 169, 171 to 176, and 182 to 192 (see Example 6 and Figure 6), and, therefore, are examples of isolated translational regulatory elements of the invention that have translational  
25 enhancing activity or IRES activity or both. In addition, examples of oligonucleotide portions of a eukaryotic mRNA 5' UTR having translational inhibitory activity include those encoded by nucleotides 120 to 196 and 167 to 196 of the Gtx homeodomain protein mRNA (SEQ ID NO: 1), and by nucleotides 1 to 100 and 100 to 160 of the yeast p150 mRNA (SEQ ID NO: 29).

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Analysis of the mouse Rbm3 5' UTR further revealed modular elements consisting of about 9 to 22 nucleotides, which exhibited IRES activity (see SEQ ID



NO: 191), translational enhancing activity (see SEQ ID NO: 192), and translational inhibitory activity (see SEQ ID NOS: 193 and 194). These results indicate that the 5' UTRs of eukaryotic mRNAs consist of a modular structure containing one or more elements that variously effect translational regulatory activity. Accordingly, the present invention also provide oligonucleotide portions of a 5' UTR of a eukaryotic mRNA that have translational enhancing activity, translational inhibitory activity, IRES activity, or a combination thereof, including, for example, SEQ ID NOS: 192 to 194.

As further disclosed, screening of libraries of random oligonucleotides containing 9 or 18 nucleotides identified numerous novel oligonucleotides having IRES activity, including combinations of such oligonucleotides. Accordingly, the present invention provides relates to a synthetic translational regulatory element, which includes at least one oligonucleotide consisting of about 6 to 125 ribonucleotides, or a deoxyribonucleotide sequence encoding the oligonucleotide. For example, the invention provide a synthetic translational regulatory element encoded by any of SEQ ID NOS: 42 to 46, 49, 50, 52, and 89 to 160, which were identified by screening libraries of randomized oligonucleotides (Example 4D). Furthermore, as disclosed herein, such oligonucleotides having translational regulatory activity can be operatively linked to each other, and can be the same or different. Examples of synthetic translational regulatory elements containing operatively linked oligonucleotides that have translational regulatory activity and are different from each other are exemplified by those encoded by any of SEQ ID NOS: 53 to 88 (see Example 4D), and examples of synthetic translational regulatory elements containing operatively linked oligonucleotides that have translational regulatory activity and are the same are exemplified by those encoded by elements containing 3 or 5 copies of SEQ ID NO: 51 or 5 copies of SEQ ID NO: 52, each copy separated by a 9 nucleotide spacer sequence (see Example 4C).

The identification of these modular IRES elements such as the 9 nucleotide Gtx IRES module, which functions independently as an IRES, is inconsistent with the hypothesis that Y-shaped RNA conformations are required for IRES activity in cellular mRNA (see, for example, Le and Maizel, *supra*, 1997). While RNA

conformation may be critical for the activity of some cellular IRESes, as it is for picornavirus IRES element, the present results suggest that RNA secondary structures may simply alter the accessibility or presentation of individual IRES modules to the translation machinery.

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A nucleotide sequence including the Gtx IRES module (SEQ ID NO: 2) directly bound to 40S ribosomal subunits by base pairing to the 18S rRNA (Hu et al., *supra*, 1999). When tested in the 5' UTR of a monocistronic reporter construct, this nucleotide sequence inhibited translation, and mutations that increased the degree of complementarity to the rRNA decreased the translation of a monocistronic mRNA, whereas mutations that decreased complementarity increased translation (Tranque et al., Proc. Natl. Acad. Sci., USA 95:12238-12243, 1998, which is incorporated herein by reference). Nucleotide sequences of the yeast YAP1 and yeast p150 5' UTRs also share complementarity with 18S rRNA sequences (see Figure 5). As disclosed herein, the Gtx IRES module (SEQ ID NO: 2) also functions as an IRES element when positioned in the intercistronic region of a dicistronic mRNA. As such, this element has opposite effects on translation, depending on whether it is present in a monocistronic mRNA or in a dicistronic mRNA. Very active IRES modules likely recruit the 40S subunit efficiently, but allow it to detach for protein synthesis to occur, whereas less active IRES modules either may recruit poorly because of weak initial interactions with the 40S subunit or may form very stable interactions that effectively sequester the mRNA.

The results disclosed herein indicate that base pairing between segments of mRNA and 18S rRNA can lead to ribosome recruitment and translation initiation, thus explaining an earlier observation that large numbers of eukaryotic mRNAs contain segments complementary to 18S rRNA (Mauro and Edelman, Proc. Natl. Acad. Sci., USA 94:422-427, 1997, which is incorporated herein by reference). If particular subsets of these mRNA sequences function as IRES modules, then large numbers of different mRNAs may use these sequences to recruit ribosomes. Sequences complementary to 18S rRNA also are present in other cellular IRESes (Gan et al., *supra*, 1998; Bernstein et al., *supra*, 1997). Similarly, the picornaviral

IRES elements contains an oligopyrimidine stretch that is complementary to the 3' end of the 18S rRNA (Scheper et al., FEBS Lett. 352:271-273, 1994). While this oligopyrimidine sequence is important for IRES activity (Meerovitch et al., J. Virol. 65:5895-5901, 1991; Pestova et al., J. Virol. 65:6194-6204, 1991), it has not been shown to base pair to the 18S rRNA. Such sequences are not found in the hepatitis C, classic swine fever, and bovine diarrhea pestivirus IRESes, although reconstitution studies provide indirect evidence for base pairing interactions between these IRESes and 18S rRNA (Pestova et al., Genes Devel. 12:67-83, 1998; Pestova and Hellen, Virology 258:249-256, 1999).

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In addition to complementarity to 18S rRNA, IRES modules also may recruit ribosomes through interactions with ribosomal proteins or with components of the translation machinery such as initiation factors, or through other intermediary proteins. For example, the iron regulatory element (IRE), which is an RNA translational control sequence, functioned as an IRES module in the presence of an engineered intermediary protein (De Gregorio et al., EMBO J. 18:4865-4874, 1999). In this system, the IRE binding protein (IRP-1), which binds the IRE, was fused to the C-terminal region of initiation factor eIF4G to produce a fusion protein that acts as an intermediary protein that can recruit the preinitiation complex, presumably through an interaction with initiation factor eIF3. This artificial recruitment of the translation machinery was sufficient to obtain weak but detectable IRES activity when the IRE was inserted in the intercistronic region of a dicistronic mRNA (De Gregorio et al., *supra*, 1999).

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The ability to internally initiate translation may be used by some mRNAs under conditions that are not favorable for cap-dependent translation, for example, during mitosis or poliovirus infection (Johannes et al., Proc. Natl. Acad. Sci., USA 96:13118-13123, 1999). However, in contrast to many viral mRNAs, virtually all eukaryotic mRNAs are monocistronic and contain a cap structure (Kozak, *supra*, 1999; Shatkin, Cell 9:645-653, 1976). Thus, the ability to internally initiate translation may be a reflection of the ability of a sequence to recruit ribosomes, and if the recruitment is sufficient to enhance translation, these sequences can confer a

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selective advantage to some mRNAs over others under competitive situations within the cell. IRES modules in eukaryotic mRNAs also can be mechanistically analogous to the prokaryotic Shine and Dalgarno sequence, albeit more complex because they are combined as multiple elements and the complementary sequences are not fixed in location either within the mRNA or rRNA.

As disclosed herein, two, five or ten copies of the 9 nucleotide Gtx IRES module (SEQ ID NO: 2) and of two synthetic oligonucleotides (SEQ ID NOS: 51 and 52) have a synergistic effect on IRES activity (Examples 1 and 4). This effect is similar to that observed with cis-acting transcriptional enhancers (Busby et al., J. Mol. Biol. 241:341-352, 1994), suggesting that the evolution of translational enhancers and regulators and may parallel that of cis-acting transcriptional enhancers. It has been suggested that the synergistic effect of transcriptional enhancers may be due to cooperative interactions between weak transcription factors, thus increasing the local concentration of these factors (Ptashne and Gann, Curr. Biol. 8:R812-R822, 1998; Ptashne and Gann, Nature 386:569-577, 1997). Although the mechanism for the observed synergistic effect of translational enhancement is not relevant to the present invention, it is noted that, similar to synergistic transcriptional enhancement, weak interactions between components of the translational machinery may explain the synergistic effects disclosed herein. Alternatively, or in addition, increased recruitment may lead to higher local concentrations of some of the initiation factors that otherwise are limiting for translation. For example, high levels of eIF4A, which is an RNA helicase, may maintain a more open mRNA secondary structure and allow more efficient translation.

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As disclosed herein, a translational regulatory element of the invention is useful for modulating the expression of a polypeptide. Synthetic IRESes of the invention were up to 63-fold more active than the EMCV IRES and appear to be much stronger than most or all of the naturally occurring IRESes that have been characterized (Borman et al., Nucl. Acids Res. 25:925-932, 1997; Martinez-Salas, Curr. Opin. Biotech. 10:458-465, 1999). In particular, methods of finely tuning the level of IRES activity by varying the number of operatively linked IRES modules are

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provided. Thus, the compositions and methods of the invention are useful, for example, for gene therapy, protein manufacturing, and the like.

5       The 5' UTR from five dendritically localized mRNAs, ARC, dendrin, MAP2, CamK IIa, and RC3, internally initiated translation when placed in the intercistronic region of a dicistronic RNA (Example 2). Furthermore, these 5' UTRs utilize both cap-independent and cap-dependent mechanisms simultaneously, resulting in the increased translation of monocistronic reporter mRNAs. Such IRES elements provide a means to ensure efficient translation of the dendritically localized transcripts.

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      A small number of mRNAs are localized to dendrites and the translation of some or all of these mRNAs is likely required to affect synaptic efficacy. Inasmuch as many of the components of the translational machinery can be limiting at these cellular sites, dendritically localized mRNAs were examined for internal ribosomal entry sites (IRES elements), which can increase translation efficiency. The 5' UTRs of the dendritically localized mRNAs were tested for IRES activity in the intercistronic region of dual luciferase dicistronic mRNAs. Transfection of neural and non-neural cell lines with constructs containing the 5' UTRs from the dendritically localized mRNAs and the encephalomyocarditis virus (EMCV) IRES resulted in translation of the second cistron that was significantly higher than that observed for a control construct containing the  $\beta$ -globin 5' UTR. Translation facilitated by these IRES elements was independent of the first cistron; translation of the second cistron occurred even when translation of the first cistron was blocked. Each of the ARC, MAP2 and CamK IIa 5' UTRs functioned as a translational enhancer in a monocistronic reporter mRNA, whereas the dendrin 5' UTR slightly inhibited translation compared to a control construct. Blocking of cap-dependent translation in these monocistronic constructs showed that internal initiation accounted for about 60% to 90% of the translation initiation of these mRNAs.

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      The ability of the dendritically localized 5' UTRs to direct internal initiation of translation was demonstrated using a dicistronic RNA containing two separately assayable luciferase reporter genes in three neural cell lines and three non-neural cell

lines (Example 2). When introduced into the intercistronic region of the dual luciferase dicistronic message, each of the 5' UTRs significantly induced translation of the second cistron. Furthermore, this activity was not inhibited by the presence of a hairpin structure at the 5' end of the dicistronic message, which effectively reduces the possibility of read-through translation from one cistron to the next, thus demonstrating that the 5' UTRs direct internal initiation of translation in a manner characteristic of an IRES. The ARC, dendrin, MAP2 and CamK IIa 5' UTRs generated significant levels of IRES activity and internally initiated translation in neural and non-neuronal cells and, therefore, can be used to modulate translation in a variety of different cell types. In addition, while the relative IRES activity generated by the 5' UTRs in non-neural cells was similar to that observed for the neural cell lines, the overall levels of IRES activity differed across cell types, and was consistently higher in non-neural cell lines.

The translation efficiency of monocistronic mRNAs is believed to be a function of the length of the 5' UTR, combined with other factors such as structure and upstream initiation codons. The  $\beta$ -globin 5' UTR is considered to be the standard of cap-dependent translation, being an optimal length, having very little secondary structure, and having no upstream AUG codons. In comparison, the lengths of the 5' UTRs for MAP2, CamK IIa and ARC average about 3 times the length of the  $\beta$ -globin 5' UTR, yet monocistronic mRNAs containing these 5' UTRs all were translated significantly better than that containing  $\beta$ -globin. However, the dendrin 5' UTR inhibited translation of monocistronic RNAs. Inhibition of translation has been observed for some viral IRES elements positioned 5' to monocistronic reporter mRNAs, and can be due to the secondary structure associated with the viral IRES inhibiting ribosomal scanning from the cap. As there is no evidence of a change in mRNA stability, the dendrin 5' UTR may have more secondary structure than the other 5' UTRs examined here. Several 5' UTRs also have been shown to be important in the developmental regulation of translation, and many of these function through IRES elements.

Since all monocistronic mRNAs should be equally capable of utilizing cap-dependent translation, the differences in translation of the monocistronic mRNAs observed herein likely is intrinsic to each 5' UTR. Since mRNA stability was not affected by the 5' UTRs (Example 2), the IRES elements contained in these 5' UTRs likely modulate translation of the monocistronic mRNAs. For the MAP2, ARC, and CamK IIa 5' UTRs, IRES activity can supplement cap-dependent translation to provide higher levels of translation. Monocistronic reporter gene mRNAs, although more physiologically relevant than dicistronic mRNAs, cannot discriminate as to whether the 5' UTRs from the dendritically localized mRNAs can employ cap-independent translational mechanisms.

The incorporation of a stable hairpin structure to prevent cap-dependent translation via ribosomal scanning of the mRNAs confirmed that all four of the 5' UTRs utilize internal initiation in monocistronic mRNAs (Example 2). In addition, the differential effects of the hairpin on the monocistronic mRNAs demonstrated that varying degrees of cap-dependent and independent mechanisms occur for each 5' UTR. For example, the dendrin 5' UTR which, unlike the other three 5' UTRs, did not increase the translation of non-hairpin monocistronic mRNA, exhibited one of the strongest effects when cap-dependent translation was inhibited. These results indicate that the dendrin 5' UTR directs translation primarily through cap-independent mechanisms; the absence of cap-dependent translation explains the observation that the dendrin 5' UTR was below control values in the monocistronic construct. The ability of a 5' UTR to effect both cap-dependent and cap-independent translation indicates that an mRNA containing such a 5' UTR is more likely to be translated than a cap-dependent mRNA such as  $\beta$ -globin, particularly under conditions where cap-dependent translation is compromised. As such, these 5' UTRs can provide a means to ensure translation of these dendritically localized mRNAs..

In viral RNAs, which lack a cap, IRES elements likely serve to circumvent cap-dependent recruitment of the translational machinery to the 5' UTR. However, since most eukaryotic mRNAs contain a cap structure, IRES elements can serve other functions in eukaryotes. In particular, a eukaryotic IRES can ensure the translation of

a particular message under conditions where cap-dependent translation is inefficient or inhibited. For several eukaryotic IRES elements that have been described, stress and other cellular states that generally hinder cap-dependent protein synthesis have little or no effect on IRES mediated translation. A similar case may occur for the  
5 dendritically localized mRNAs, which, as disclosed herein, contain 5' UTRs direct cap-dependent and cap-independent translation (Example 2). Furthermore, cap-dependent translation is tightly regulated by cellular events and often involves modulation of cap-binding activity. As such, an mRNA containing an IRES can be translated efficiently during a cellular stress condition such as ischemia or apoptosis,  
10 which otherwise affects overall levels of translation.

Experiments using a dominant negative form of the eukaryotic initiation factor-4 (eIF4) binding protein, eIF4-bp1, demonstrated that the 5' UTRs from the dendritically localized mRNAs initiated translation under conditions where cap  
15 binding is inhibited. Endogenous 4E-BP1 binds to and sequesters eIF4E, disabling cap-dependent translation until 4E-BP1 is phosphorylated. The 4E-BP1 mutant, which lacks the critical phosphorylation sites necessary for disengagement with eIF4E, irreversibly binds eIF4E and prevents cap-dependent translation. Transfection of this mutant 4E-BP1 with monocistronic 5' UTR constructs reduced the cap-  
20 dependent translation from the 5' UTR of  $\beta$ -globin by greater than 55% (lack of further reduction of  $\beta$ -globin can be related to plasmid titer, lack of co-transfection in some cells, or feedback on the 4E-BP1 mutant's own capped message), whereas constructs containing the 5' UTRs initiated translation of monocistronic mRNAs to various degrees in the absence of cap-binding. The lack of dependence of dendrin 5'  
25 UTR on cap-binding for translation supports the previous observation that this 5' UTR is primarily translated through cap-independent mechanisms. These results indicate that IRES mediated translation can yield constitutive expression of certain proteins, regardless of cap-dependent translational control. Furthermore, the degree to which the IRES can internally initiate translation in the absence of cap-binding provides an  
30 additional level of translational regulation, including providing a means for maintaining translation of cellular messages critical for important cellular events.



Although it is not clear whether cap-independent mechanisms are utilized within the dendritic compartment, each of the five 5' UTRs, which were selected based on their mRNA subcellular distribution, demonstrated IRES activity to some degree. Remarkably, these 5' UTRs do not share characteristics of other IRES elements, were not selected, for example, based on length, exceptional GC content, or excess of upstream AUG start codons, and have no overall sequence similarity. The 5' UTR from the neural cell adhesion molecule (NCAM) mRNA, a neuronal message that is not dendritically localized, did not demonstrate any significant IRES activity. It has been reported that cap-analogs, which prevent cap-dependent protein synthesis, can perturb lasting changes in long-term depression. Those observation together with the results disclosed herein suggest that dendrites may utilize internal initiation as a mechanism for translation of dendritically localized messages, and may do so in response to synaptic stimuli or other perturbations in cellular homeostasis.

As disclosed herein, a translational regulatory element of the invention can be contained in a vector. The vector can be any vector, but preferably is an expression vector, which can contain a translation initiation site in addition to the translational regulatory element. Generally, the translational regulatory element will have translational enhancing activity or IRES activity, particularly where the vector is to further contain an expressible polynucleotide. However, the vector also can contain a translational regulatory element of the invention having translational inhibitory activity, for example, where it is desired to introduce the element into a cell to inhibit translation of a polypeptide to which it is operatively linked, or where it is desired to use the element to bind translational regulatory factors that would otherwise bind an endogenous form of such an element and inhibit translation.

Where the vector is an expression vector, it can further contain a translation start codon, which can be operatively linked to a translation initiation site and to the translational regulatory element. However, such elements also can be part of an expressible polynucleotide that is to be inserted into the vector. Accordingly, a vector of the invention can contain an expressible polynucleotide, wherein the translational regulatory element is operatively linked to the expressible polynucleotide. As used

herein, the term "expressible polynucleotide" refers to any polynucleotide that can be transcribed into an RNA molecule and, particularly, can be translated into a polypeptide. The expressible polynucleotide can simply be a coding sequence of a polypeptide, or can contain one or more regulatory elements generally associated with a polynucleotide to be transcribed or translated, for example, a transcriptional promoter, transcription start site, polyadenylation signal sequence, Kozak sequence, initiator methionine codon, stop codon and the like. Where the expressible polynucleotide contains all or substantially all of the elements required for expression of a polypeptide, it also is referred to herein as a "cistron". In referring to "substantially" all of the element required for expression, it will be recognized that a cistron may or may not contain a translational regulatory element, since such an element is provided according to the present invention.

It should further be recognized that an expressible polynucleotide can contain more than one cistron, for example, can be dicistronic such as the numerous dicistronic expressible polynucleotide described in the Examples. In general, where an expressible polynucleotide contains more than one cistron, the cistrons are operatively linked such that they are transcribed as a single RNA molecule. In addition, a spacer nucleotide sequence generally will be present between the cistrons, and, if desired, a translational regulatory element of the invention having IRES activity or translational enhancing activity or both can be inserted into the spacer nucleotide sequence. As disclosed herein, an expressible polynucleotide can be contained in a vector of the invention.

A vector of the invention also can contain additional elements generally found in a vector, including, for example, a nucleotide sequence encoding a polypeptide that confers antibiotic resistance upon a cell containing the vector, including a bacterial cell, an insect cell, or a mammalian cell, as desired, and can contain an origin of replication, for example, a bacterial origin of replication such that the vector can be amplified in a bacterial host cell. Accordingly, the invention also provides a host cell containing a vector of the invention. Vectors, which include plasmid vectors and viral vectors such as bacteriophage, baculovirus, retrovirus, lentivirus, adenovirus, vaccinia virus, semliki forest virus and adeno-associated virus vectors, and host cells for containing a particular vector, are well known and can be purchased from a commercial source

(Promega, Madison WI; Stratagene, La Jolla CA; Invitrogen, La Jolla CA) or can be constructed by one skilled in the art (see, for example, Meth. Enzymol., Vol. 185, Goeddel, ed. (Academic Press, Inc., 1990); Jolly, Canc. Gene Ther. 1:51-64, 1994; Flotte, J. Bioenerg. Biomemb. 25:37-42, 1993; Kirshenbaum et al., J. Clin. Invest. 5 92:381-387, 1993; each of which is incorporated herein by reference).

Where a translational regulatory element of the invention is to be introduced into a cell in culture or in an organism, a viral vector can be particularly useful. Viral vectors provide the advantage that they can infect host cells with relatively high efficiency and can infect specific cell types. For example, a translational regulatory element of the invention operatively linked to an expressible polynucleotide that is to be expressed in lung cells can be cloned into an adenovirus vector, which effectively infects lung epithelial cells. Similarly, a translational regulatory element of the invention operatively linked to an expressible polynucleotide that is to be expressed in neuronal cells can be cloned into a herpesvirus vector, which effectively infects neuronal cells. Viral vectors have been developed for use in particular host systems, particularly mammalian systems and include, for example, retroviral vectors, other lentivirus vectors such as those based on the human immunodeficiency virus (HIV), adeno-associated virus vectors, herpesvirus vectors, vaccinia virus vectors, and the like (see Miller and Rosman, BioTechniques 7:980-990, 1992; Anderson et al., Nature 10 392:25-30 Suppl., 1998; Verma and Somia, Nature 389:239-242, 1997; Wilson, New Engl. J. Med. 334:1185-1187 (1996), each of which is incorporated herein by reference).

25 A translational regulatory element of the invention, which can be contained in a vector, can be introduced into a cell by any of a variety of methods known in the art (Sambrook et al., Molecular Cloning: A laboratory manual (Cold Spring Harbor Laboratory Press 1989); Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, MD (1987, and supplements through 1995), each of which is incorporated herein by reference). Such methods include, for example, transfection, lipofection, microinjection, electroporation and, with viral vectors, infection; and can include the use of liposomes, microemulsions or the like, which can 30

facilitate introduction of the polynucleotide into the cell and can protect the polynucleotide from degradation prior to its introduction into the cell. The selection of a particular method will depend, for example, on the cell into which the translational regulatory element is to be introduced, as well as whether the cell is  
5 isolated in culture, or is in a tissue or organ in culture or *in situ*, and, as disclosed herein, the selection system can be based on a reporter polypeptide that is expressed, at least in part, due to the translational regulatory element of the invention. Introduction of a translational regulatory element of the invention into a cell by infection with a viral vector is particularly advantageous in that it can efficiently  
10 introduce the nucleic acid molecule into a cell *ex vivo* or *in vivo* (see, for example, U.S. Pat. No. 5,399,346, which is incorporated herein by reference).

The present invention further relates to a recombinant nucleic acid molecule, which contains a translational regulatory element of the invention operatively linked to  
15 an expressible polynucleotide. The expressible polynucleotide can comprise a cistron, and the translational regulatory element have translational enhancing activity or IRES activity or both. The expressible polynucleotide also can comprises, in operative linkage in a 5' (upstream) to 3' (downstream) orientation, a first cistron, a spacer nucleotide sequence, and a second cistron, and the translational regulatory element can have IRES  
20 activity, and be operatively linked to the second cistron. The synthetic translational regulatory element in a recombinant nucleic acid molecule of the invention can include two or more operatively linked oligonucleotides having translational regulatory activity, for example, ten copies of SEQ ID NO: 2, each of which can be separated by a spacer nucleotide sequence.

25

The polypeptide encoded by a recombinant nucleic acid molecule of the invention can be any polypeptide, including, for example, an enzyme such as  $\beta$ -galactosidase,  $\beta$ -glucuronidase, luciferase, alkaline phosphatase, glutathione S-transferase, chloramphenicol acetyltransferase, guanine xanthine  
30 phosphoribosyltransferase, and neomycin phosphotransferase; a viral or bacterial polypeptide or an epitope expressed by a virus, bacterium or other pathogenic organism; or a growth factor, a hormone and a receptor for a growth factor or a

hormone. The recombinant nucleic acid molecule also can encode two or more polypeptides, for example, a first polypeptide that encodes a reporter polypeptide, which can be a selectable marker, and a second polypeptide encoding a polypeptide of interest, for example a therapeutic polypeptide.

5

The term "oligonucleotide", "polynucleotide" or "nucleotide sequence" is used broadly herein to mean a sequence of two or more deoxyribonucleotides or ribonucleotides that are linked together by a phosphodiester bond. As such, the terms include RNA and DNA, which can be a gene or a portion thereof, a cDNA, a synthetic polydeoxyribonucleic acid sequence or polyribonucleic acid sequence, or the like, and can be single stranded or double stranded, as well as a DNA/RNA hybrid. Furthermore, the terms "oligonucleotide", "polynucleotide" and "nucleotide sequence" include naturally occurring nucleic acid molecules, which can be isolated from a cell, as well as synthetic molecules, which can be prepared, for example, by methods of chemical synthesis or by enzymatic methods such as by the polymerase chain reaction (PCR).

For convenience of discussion, the term "oligonucleotide" generally is used to refer to a nucleotide sequence that has translational regulatory activity, particularly a nucleotide sequence of a translational regulatory element of the invention. In comparison, the term "polynucleotide" or "nucleotide sequence" generally refers to a sequence that encodes a peptide or polypeptide, acts as or encodes a desired regulatory element, provides a spacer sequence or cloning site, or the like. It should be recognized, however, that such a use only is for convenience and is not intended to suggest any particular length or other physical, chemical, or biological characteristic of the nucleic acid molecule.

Synthetic methods for preparing a nucleotide sequence include, for example, the phosphotriester and phosphodiester methods (see Narang et al., Meth. Enzymol. 68:90, (1979); U.S. Pat. No. 4,356,270, U.S. Pat. No. 4,458,066, U.S. Pat. No. 4,416,988, U.S. Pat. No. 4,293,652; and Brown et al, Meth. Enzymol. 68:109, (1979), each of which is incorporated herein by reference). In various embodiments,

an oligonucleotide of the invention or a polynucleotide useful in a method of the invention can contain nucleoside or nucleotide analogs, or a backbone bond other than a phosphodiester bond. The nucleotides comprising an oligonucleotide (polynucleotide) generally are naturally occurring deoxyribonucleotides, such as adenine, cytosine, guanine or thymine linked to 2'-deoxyribose, or ribonucleotides such as adenine, cytosine, guanine or uracil linked to ribose. However, a polynucleotide also can contain nucleotide analogs, including non-naturally occurring synthetic nucleotides or modified naturally occurring nucleotides. Such nucleotide analogs are well known in the art and commercially available, as are polynucleotides containing such nucleotide analogs (Lin et al., Nucl. Acids Res. 22:5220-5234 (1994); Jellinek et al., Biochemistry 34:11363-11372 (1995); Pagratis et al., Nature Biotechnol. 15:68-73 (1997), each of which is incorporated herein by reference).

The covalent bond linking the nucleotides of an oligonucleotide or polynucleotide generally is a phosphodiester bond. However, the covalent bond also can be any of numerous other bonds, including a thiodiester bond, a phosphorothioate bond, a peptide-like bond or any other bond known to those in the art as useful for linking nucleotides to produce synthetic polynucleotides (see, for example, Tam et al., Nucl. Acids Res. 22:977-986 (1994); Ecker and Crooke, BioTechnology 13:351360 (1995), each of which is incorporated herein by reference). The incorporation of non-naturally occurring nucleotide analogs or bonds linking the nucleotides or analogs can be particularly useful where the nucleotide sequence is to be exposed to an environment that can contain a nucleolytic activity, including, for example, a tissue culture medium or upon administration to a living subject, since the modified nucleotide sequences can be less susceptible to degradation.

A polynucleotide comprising naturally occurring nucleotides and phosphodiester bonds can be chemically synthesized or can be produced using recombinant DNA methods, using an appropriate polynucleotide as a template. In comparison, a polynucleotide comprising nucleotide analogs or covalent bonds other than phosphodiester bonds generally are chemically synthesized, although an enzyme such as T7 polymerase can incorporate certain types of nucleotide analogs into a

polynucleotide and, therefore, can be used to produce such a polynucleotide recombinantly from an appropriate template (Jellinek et al., *supra*, 1995).

A recombinant nucleic acid molecule, or a vector, of the invention can contain  
5 additional sequences that confer a desired characteristic on the polynucleotide. In particular, the recombinant nucleic acid molecule or vector can contain an operatively linked transcriptional regulatory element, including a transcriptional promoter or enhancer, which can be a constitutively expressed regulatory element, which maintains expression of a polynucleotide at a relatively constant level of activity  
10 (basal level), or can be an inducible regulatory elements. Constitutively expressed regulatory elements can be expressed in any cell type, or can be tissue specific, which are expressed only in particular cell types, phase specific, which are expressed only during particular developmental or growth stages of a plant cell, or the like. A regulatory element such as a tissue specific or phase specific regulatory element or an  
15 inducible regulatory element useful in constructing a recombinant polynucleotide or in practicing a method of the invention can be a regulatory element that generally, in nature, is found in a plant genome. However, the regulatory element also can be from an organism other than a plant, including, for example, from a plant virus, an animal virus, or a cell from an animal or other multicellular organism.

20

As disclosed herein, translational regulatory elements can be selected based on their ability to confer tissue specific translation, or translation in response to an inducing agent. As used herein, the term "tissue specific," when used in reference to a translational regulatory element, means a nucleotide sequence that effects translation  
25 of an operatively linked expressible polynucleotide in only one or a few cell types. As used herein, the term "inducible," when used in response to a translational regulatory element, means a nucleotide sequence that, when present in a cell exposed to an inducing agent, effects an increased level of translation of an operatively linked expressible polynucleotide as compared to the level of translation, if any, in the  
30 absence of an inducing agent.

The term "inducing agent" is used to refer to a chemical, biological or physical agent that effects translation from an inducible translational regulatory element. In response to exposure to an inducing agent, translation from the element generally is initiated *de novo* or is increased above a basal or constitutive level of expression.

5 Such induction can be identified using the methods disclosed herein, including detecting an increased level of a reporter polypeptide encoded by the expressible polynucleotide that is operatively linked to the translational regulatory element. An inducing agent can be, for example, a stress condition to which a cell is exposed, for example, a heat or cold shock, a toxic agent such as a heavy metal ion, or a lack of a  
10 nutrient, hormone, growth factor, or the like; or can be exposure to a molecule that affects the growth or differentiation state of a cell such as a hormone or a growth factor. By examining the translational regulatory activity of a translational regulatory element of the invention or a combination of such elements in cells exposed to such conditions, or cells of a particular cell type, translational regulatory elements that are  
15 inducible or tissue specific can be identified. As such, a recombinant nucleic acid molecule of the invention can contain a tissue specific or inducible translational regulatory element.

An expressible polynucleotide can be any polynucleotide that is operatively  
20 linked to a translational regulatory element of the invention and encodes a polypeptide. As used herein, the term "operatively linked" means that a regulatory element, which can be a transcriptional or translational regulatory element, including a translational regulatory element of the invention, is positioned with respect to a transcribable or translatable nucleotide sequence such that the regulatory element can effect its  
25 regulatory activity. A transcriptional enhancer, for example, can be located at any distance, including adjacent to or up to thousands of nucleotides away from, and upstream or downstream from the promoter and nucleotide sequence to be transcribed, and still exert a detectable effect on the level of expression of an encoded reporter molecule. In comparison, a translational regulatory element generally is  
30 contained within about 1 to 500 nucleotides, particularly within about 1 to 100 nucleotides of a translation start site. In addition, the term "operatively linked" is used with respect to a first and second polypeptide (or peptide) to refer to encoding



sequences that are linked in frame such that a fusion polypeptide can be produced. Similarly, the term is used to refer to two or more cistrons of an expressible polynucleotide that are transcribed as a single RNA molecule, which can contain, for example, an IRES element of the invention in an intercistronic position.

5

As used herein, the term "heterologous" is used in a comparative sense with respect to a nucleotide sequence to indicate either that the nucleotide sequence is not an endogenous nucleotide sequence in a cell into which it is to be introduced, or that the nucleotide sequence is part of a construct such that it is in a form other than it normally would be found in a cell. For example, a heterologous nucleotide sequence can be a polynucleotide that encodes a mammalian viral polypeptide, where the heterologous nucleotide sequence is to be introduced into a plant cell; i.e., the nucleotide sequence is heterologous with respect to the plant cell. A heterologous nucleotide sequence also can be, for example, a portion of a plant gene sequence that is operably linked to a plant CRRE, provided that the construct is not normally found in a plant cell, for example, a plant CRRE operably linked to a heterologous nucleotide sequence consisting of a hypothetical plant gene exon 3, wherein the plant gene can be a circadian-regulated gene comprising the plant CRRE to which the exon is operably linked. Thus, a heterologous nucleotide sequence in a recombinant polynucleotide of the invention can be any nucleotide sequence that is not normally part of the plant circadian-regulated gene from which the oligonucleotide comprising the CRRE-component of the recombinant polynucleotide is obtained; or, if it is a part of the plant circadian-regulated gene from which the CRRE is obtained, it is an orientation other than it would normally be in the gene, for example, is an antisense sequence, or comprises at least a partially discontinuous sequence as compared to the genomic structure.

The present invention also relates to a kit, which contains a translational regulatory element of the invention. In particular, a kit of the invention can contain a plurality of synthetic transcriptional regulatory elements, particularly a plurality of different translational regulatory element. The translational regulatory elements of the plurality can be used individually for a specific purpose depending on the characteristics of the element, for example, translational enhancing activity, IRES activity, or

translational inhibitory activity, or tissue specific expression, inducible expression, or other characteristic. In addition, the translational regulatory elements of the plurality can be used in combination to provide, for example, a high level of translational enhancing or IRES activity.

5           The translational regulatory elements in a kit of the invention can contain one or more flanking sequence, which, independently, can be at a 5' end or a 3' end or both 5' and 3' ends. Such flanking sequences can be, for example, restriction endonuclease recognition sites or recombinase recognition sites such as a lox or att sequence, and can be single stranded overhangs. As such, the flanking sequence can be used to facilitate  
10           operatively linking two or more translational regulatory elements in the plurality to each other or for linking the element to any other polynucleotide.

          The present invention also relates to a method of producing a genetically modified cell that exhibits altered expression of a polypeptide. The method can be  
15           performed, for example, by introducing a translational regulatory element of the invention into a cell, whereby the synthetic translational regulatory element operatively linked to a nucleotide sequence encoding a polypeptide. As disclosed herein, the translational regulatory element can be operatively linked to the nucleotide sequence, which can be an expressible polynucleotide, prior to introducing the  
20           translational regulatory element into the cell. For example, the translational regulatory element can have translational enhancing activity or IRES activity such that the encoded polypeptide is expressed in the cell, preferably in a desired level. The polypeptide can be any polypeptide, including, for example, a reporter polypeptide, a toxin, or a therapeutic agent.

25

          As disclosed herein, the nucleotide sequence to which the translational regulatory element is operatively linked also can be a sequence of an endogenous gene in the cell genome. Where it is desired, for example, to decrease the expression of an endogenous polypeptide in a cell, for example, expression of an activated Ras  
30           protein, the translational regulatory element is selected based on its having translational inhibitory activity. The translational regulatory element can be targeted to a particular locus, for example, upstream of the start codon of a mutant Ras gene

using a method such as homologous recombination. In addition, a translational regulatory element having translational inhibitory activity also can be introduced into a eukaryotic cell such that it can randomly integrate into the cell genome, thus providing a novel means to obtain a "knock out" phenotype in a cell.

5

The production of a genetically modified cell expressing a heterologous polypeptide, for example, is facilitated by including the nucleotide sequence of interest as one cistron of a polycistronic expressible polynucleotide, to which one or more translational regulatory elements of the invention, including one or more IRES elements is operatively linked. One or more other cistrons in the expressible polynucleotide can encode, for example, a reporter molecule such that cells containing the construct can be identified, selected and isolated. One or more other cistrons in the construct also can encode a second polypeptide that effects, for example, the level of transcription of the expressible polynucleotide in the genetically modified cell, or the activity of the expressed heterologous polypeptide in the cell.

Accordingly, the present invention also relates to a genetically modified eukaryotic cell produced by a method of the invention, as well as to a transgenic non-human eukaryotic organism containing or derived from such a genetically modified cell, a cell or tissue obtained from the transgenic non-human eukaryotic organism, and a cDNA or genomic DNA library prepared from the transgenic non-human eukaryotic organism or from a cell or tissue obtained from said transgenic non-human eukaryotic organism. Such a genetically modified cell or transgenic organism can be useful, for example, to produce a polypeptide of interest, or as a model system for a pathologic condition, for example, a condition characterized in part by overexpression or accumulation of a particular polypeptide.

The present invention further provides a method of altering translational activity in a eukaryotic cell, the method comprising introducing into the cell a synthetic translational regulatory element. As disclosed herein, introduction of a translational regulatory element of the invention having translational enhancing activity can reduce the level of translation in a cell. While no mechanism for this action is proposed herein

or, in fact, relevant to practicing a method of the invention, one possibility is that the element can bind to and sequester trans-acting translational regulatory factors such as eukaryotic initiation factors or the like, similar to effects seen with transcriptional regulatory elements when introduced into cells. Thus, by introducing a translational regulatory element of the invention having translational enhancing activity or IRES activity into a eukaryotic cell, the translational activity in the eukaryotic cell can be reduced or inhibited. Conversely, by introducing a translational regulatory element having translational inhibitory activity into a eukaryotic cell, translational activity in the cell is increased due, for example, to the sequestering of a trans-acting factor that otherwise binds to an endogenous translational inhibitory sequence in the cell to inhibit translation.

The present invention also provides a means to conveniently and efficiently select specific cells and cell populations. Identification of such cells is effected by introducing a construct containing a translational regulatory element, for example, an IRES element operatively linked to a nucleotide sequence encoding a reporter molecule such as a fluorescent protein or selectable marker. Where an IRES element is used, the IRES element can drive expression of a monocistronic reporter cassette or can drive expression of a second or other cistrons in a polycistronic cassette, one or more of which encode a reporter polypeptide and one or more others of which can encode any polypeptide or polypeptides of interest. Generally, in a polycistronic reporter, an IRES element is contained in an intercistronic position, particularly upstream of the cistron to be regulated by the IRES. Thus, desired cells can be selected based on expression of the reporter polypeptide driven by the translational regulatory element, and, if desired, the cell can be one that also expresses a heterologous polypeptide of interest, which can be co-expressed from the same construct as the reporter polypeptide.

Particularly useful co-expressed reporter polypeptides can include cell surface polypeptides, which act as markers that permit immunocytometric or affinity chromatographic selection and isolation of the cells expressing the protein of interest; enzymes or other polypeptides that confer antibiotic resistance, which allow the use of

a growth selection method to identify, select and isolate cells co-expressing the protein of interest; other enzymes that confer a unique reporting capability to a cell, for example, alkaline phosphatase, horseradish peroxidase or any other enzyme generally used for as a histologic or diagnostic reporter molecule. Such polypeptides, including a reporter polypeptide and co-expressed protein of interest can be expressed under *in vivo* or *in vitro* conditions and, similarly, the cells expressing the polypeptide can be identified, selected, or isolated under *in vivo* or *in vitro* conditions.

The present invention also provides methods for improving protein yields by a cell, which can be a cell in culture or a cell in an organism, through enhanced protein stability, enhanced cell stability and viability, and the like. Stability of expressed protein products can be improved using, for example, IRES mediated co-expression of proteins or peptides such as chaperone proteins, heat shock proteins, proteins such as superoxide dismutase and catalase, which decrease the levels of oxidizing agents in a cell, protease inhibitors, phosphatase inhibitors, and the like, which can enhance the stability of the protein of interest or a cell expressing the protein of interest, thereby enhancing the yield of the protein obtainable from the cell. For example, co-expression of protease inhibitors with a protein of interest can protect the protein from cellular proteases, thus prolonging the time the protein is present in a cell and increasing the yield obtainable from the cell. Similarly, enzyme inhibitors or modulators such as caspase inhibitors can maintain cellular integrity during expression of the protein of interest by prolonging the life of the cell. Antibiotics peptides such as magainins, defensins, cryptdins and the like also can be co-expressed with the protein of interest, thereby protecting the cell from microbial contamination, thus increasing the likelihood that the cells can be maintained in a healthy and productive state. Furthermore, by using concatemers of the disclosed translational regulatory elements, including concatemers of the same or different elements, very high levels of translation can be obtained and translation in particular cell types or at particular stages of the cell cycle can be obtained. Such methods are particularly valuable to the bioprocessing industry, which is involved in large scale manufacture of proteins for medical and industrial use.

Improved protein yields also can be obtained by using an IRES element for co-expressing a transcription factor specific for a promoter element contained in the construct comprising the IRES element, or for co-expressing translational regulatory factors such as translation eukaryotic initiation factors that are specific for the IRES or other translational regulatory elements contained in the construct. Such methods provide a means for enhancing cellular signaling in the environment of the cell producing the target protein. IRES mediated co-expression of agents that affect the cellular environment of the cell expressing a target protein can be particularly useful for *in vivo* diagnostic and therapeutic applications, as well as for *in vitro* bioproduction methods and as research tools. For example, the co-expressed agents can include proteins or peptides from the classes of neuromodulators, peptide/protein hormones, growth factors, adhesion proteins, cell media peptides, and the like.

The present invention also provides methods for producing complex proteins or protein systems. For example, IRES-mediated co-expression of the subunits of a multi-component protein has been reported (see, for example, U.S. Pat. No. 6,060,273). However, unique applications using the presently disclosed translational regulatory elements, including IRES elements, allow the co-expression of enzymes or pro-enzymes with specific substrates, prosthetic groups, allosteric modulators, processing enzymes, chaperones, and the like.

The following examples are intended to illustrate but not limit the invention.

#### EXAMPLE 1

##### **IDENTIFICATION OF A MODULAR IRES ELEMENT THAT ENHANCES TRANSLATIONAL ACTIVITY**

This example describes the identification and characterization of a nine nucleotide IRES element from the 5' untranslated region (5' UTR) of the mRNA encoding the Gtx homeodomain protein.

### **A. Identification of a Nine Nucleotide IRES Element in the Gtx 5' UTR**

Dicistronic constructs were based on the pGL3-R2 (RP) and pGL3-R2h (RPh) dicistronic reporter vectors (Chappell et al., Proc. Natl. Acad. Sci., USA 97:1536-1541, 2000, each of which is incorporated herein by reference; see, also, Stoneley et al., *supra*, 1998). These vectors encode a dicistronic mRNA, with the *Renilla* (sea pansy) luciferase gene upstream of the *Photinus* (firefly) luciferase gene. An SV40 promoter and enhancer drive expression. The RPh vector contains a 60 bp inverted repeat located 5' (upstream) of the first cistron. The 5' UTR of the Gtx mRNA (SEQ ID NO: 1; Komuro et al., *supra*, 1993) was synthesized as two overlapping oligonucleotides, amplified by PCR with Pfu DNA polymerase (Stratagene, La Jolla CA), and inserted into the intercistronic region as an Eco RI to Nco I fragment immediately upstream of the *Photinus* luciferase initiation codon in both the RP and RPh vectors. Other inserts were similarly cloned into the intercistronic region using the Eco RI and Nco I restriction sites, or using the Spe I and Eco RI restriction sites. The sequences of all constructs were verified. Plasmids containing the chloramphenicol acetyl transferase (CAT) gene instead of the *Photinus* luciferase gene were derived by deleting the luciferase gene and replacing it with the CAT gene using the Nco I and Bam HI restriction sites (RCh). The CAT gene was from the pCAT3 control vector (Promega; Madison WI).

20

Cell lines were obtained from ATCC and maintained according to their instructions. Dicistronic reporter constructs were transfected into cells using FuGENE 6 reagent (Roche; Indianapolis IN), according to the manufacturer's instructions. Transfection efficiencies were normalized by cotransfection with the pCMV $\beta$  vector (Clontech; Palo Alto CA). Cells were harvested after 24 hr and luciferase activity was determined using the dual reporter assay system (Promega).  $\beta$ -galactosidase activity was assayed using the Fluoreporter lacZ kit (Molecular Probes; Eugene OR) and fluorescence was measured using the Millipore Cytofluor 2450 system. CAT enzyme activity was measured using n-butyryl CoA according to technical bulletin #84 (Promega).

30

Northern blot analysis was performed as described by Mauro and Edelman (*supra*, 1997) using RNA prepared by the guanidinium thiocyanate method from the neuronal cell line Neuro 2a (N2a; Chomczynski and Sacchi, Anal. Biochem. 162:156-159, 1987, which is incorporated by reference). Poly(A)<sup>+</sup> RNA was selected  
5 using Oligotex oligo(dT) beads (Qiagen; Chatsworth CA). Hybridizations were performed using Rapid hyb buffer (Amersham; Piscataway NJ) with a probe containing the entire *Photinus* luciferase coding sequence. This probe was obtained by digesting the dicistronic construct at the Nco I and Sal I restriction sites. Hybridizations were performed at 65°C; wash stringency was 65°C in 0.1X saline  
10 sodium citrate (SSC).

The 5' UTR of the Gtx mRNA (SEQ ID NO: 1) is 196 nucleotides in length (Komuro et al., *supra*, 1993; see, also, GenBank Accession No. L08074, ATG is at nucleotides 197 to 199) and contains several complementary sequence matches to  
15 18S rRNA, one of which was the focus of a previous study (Hu et al., *supra*, 1999). In order to determine whether a sequence of 5' UTR can interact directly with complementary sequences in the 18S rRNA, the Gtx 5' UTR was inserted into the intercistronic region of the dicistronic mRNA (Gtx<sub>1-196</sub>/RP), which contains the *Renilla* luciferase coding sequence as the first cistron and the *Photinus* luciferase  
20 coding sequence as the second cistron (Stoneley et al., *supra*, 1998). The presence of the 196 nucleotide Gtx 5' UTR enhanced expression of the downstream cistron about 7.2-fold over background in the rat neuronal N2a cell line and about 8.8-fold in the rat glioma C6 cell line. The IRES activity was approximately 47% of that of the encephalomyocarditis virus (EMCV) IRES in N2a cells and 84% of that of EMCV in  
25 C6 cells when tested in the same vector system (EMCV/RP). Translation of the β-globin mRNA is cap-dependent (Keiper and Rhoads, Nucl. Acids Res. 25:395-402, 1997). As such, insertion of the 5' UTR of the β-globin mRNA in the intercistronic region served as a control (no IRES activity; β-globin/RP).

30 In order to confirm that enhanced expression of the second cistron was not dependent on translation of the first cistron, translation of the first cistron was inhibited by introducing an inverted repeat, which has potential to form a stable



hairpin structure, in the 5' UTR (Gtx<sub>1-196</sub>/RPh). When normalized to the activity of the RP vector, the presence of the hairpin structure resulted in increased IRES activity up to 31-fold over background. Northern blot analysis using poly(A)<sup>+</sup> RNA isolated from N2a cells transfected with the Gtx<sub>1-196</sub>/RP vector and probed with a *Photinus* luciferase fragment showed that the cells expressed only a single detectable mRNA of the correct expected size. These results demonstrate that enhanced expression of the downstream *Photinus* luciferase gene was not due to the generation of a functional monocistronic mRNAs due, for example, to an unusual splicing event or RNA fragmentation.

10

In order to determine whether the Gtx 5' UTR contains shorter segments with IRES activity, the 5' UTR was sequentially deleted from the 5' and 3' ends, and was fragmented into 5 non-overlapping segments of approximately 40 nucleotides (see Figure 1), each of which was tested for IRES activity. To minimize the contribution of the first cistron, the RPh vector was used for these constructs. All of the deleted and fragmented sequences examined had IRES activity, with the exception of constructs Gtx<sub>120-196</sub>/RPh and Gtx<sub>167-196</sub>/RPh. Deletion of nucleotides 167 to 196 of SEQ ID NO: 1 increased IRES activity to a level greater than that of the entire 5' UTR (Gtx<sub>1-166</sub>/RPh and Gtx<sub>120-166</sub>/RPh). These results indicate that either the 167 to 196 region of SEQ ID NO: 1 inhibits IRES activity, or the IRES activity exhibited by nucleotides 1 to 166 of SEQ ID NO: 1 is dependent on its location relative to the initiation codon.

20

The nucleotide sequence 133 to 141 (CCGGCGGGT; SEQ ID NO: 2), which is contained within the Gtx<sub>120-166</sub>/RPh construct, is 100% complementary to nucleotides 1332 to 1124 of 18S rRNA, and has been shown to crosslink to its complement within intact 40S subunits (Hu et al., *supra*, 1999). The 9 nucleotide Gtx sequence (SEQ ID NO: 2) was directly tested for IRES activity. The spacing of the 9 nucleotide Gtx sequence relative to the initiation codon was maintained as in the Gtx<sub>120-166</sub>/RPh construct by using a polynucleotide sequence identical to the whole  $\beta$ -globin 5' UTR (SEQ ID NO: 3), which lacks detectable IRES activity. IRES activity associated with this construct (see SEQ ID NO: 4) was about 3.4-fold over the

30

background. These results demonstrate that a core 9 nucleotide sequence of the Gtx 5' UTR (SEQ ID NO: 2) has IRES activity. As such, this sequence is referred to as the Gtx IRES module (SEQ ID NO: 2).

#### 5 **B. Operatively Linked IRES Modules Enhance Translation Synergistically**

Dicistronic constructs were generated containing 1 (SEQ ID NO: 5), 5 (SEQ ID NO: 6), or 10 (SEQ ID NO: 7) copies of the Gtx IRES module (SEQ ID NO: 2). The IRES modules were positioned beginning 25 nucleotides upstream of the initiation codon using  $\beta$ -globin 5' UTR sequences (see SEQ ID NOS: 5 to 7).

10 Constructs with 5 or 10 copies of the IRES module were arbitrarily spaced 9 nucleotides from each other using a repeated segment of the  $\beta$ -globin 5' UTR (designated SI; see, for example, SEQ ID NO: 6). In N2a cells, IRES activity of the Gtx<sub>133-144</sub>/RPh construct, which contained a single copy of the IRES module (SEQ ID NO: 5), was 2.5-fold over background; IRES activity of the (Gtx<sub>133-144</sub>)<sub>5</sub>(SI)<sub>5</sub>/RPh

15 construct (5 copies; SEQ ID NO: 6) was 164-fold over background; and IRES activity of the (Gtx<sub>133-144</sub>)<sub>10</sub>(SI)<sub>9</sub> $\beta$ /RPh construct (10 copies; SEQ ID NO: 7) was 570-fold over background (IRES activities were normalized to activity of the parent RPh vector). In comparison, when normalized to the activity of the RP vector, IRES activity of the (Gtx<sub>133-144</sub>)<sub>10</sub>(SI)<sub>9</sub> $\beta$ /RPh construct was about 6,000-fold over background.

20

The ratio of *Photinus* luciferase activity to *Renilla* luciferase activity (P:R ratio) reflects the absolute increase in *Photinus* luciferase activity for all constructs except those that contain 5 or 10 copies of the Gtx<sub>133-144</sub> sequence with the SI spacer, in which the expression of both cistrons increased. The level of *Renilla* luciferase

25 expression obtained with the (Gtx<sub>133-144</sub>)<sub>10</sub>(SI)<sub>9</sub> $\beta$ /RPh construct was approximately 20-fold higher than that of construct RPh. Northern blot analysis of mRNAs from N2a cells indicated that the mRNA levels were not significantly different. Thus, it is unlikely that increased mRNA levels were responsible for the increased expression of the *Renilla* gene. A similar enhancement of both cistrons was observed when the

30 tobacco necrosis virus enhancer was placed in the 3' UTR of a dicistronic mRNA (Meulewaeter et al., RNA 4:1347-1356, 1998).

Two other spacer sequences and a number of controls were examined to distinguish between the effects of the 9 nucleotide IRES module (SEQ ID NO: 2) and the spacer sequence. One spacer sequence included a different 9 nucleotide segment of the  $\beta$ -globin 5' UTR (designated SII; see SEQ ID NOS: 8 and 9) and the other  
5 included a poly(A)<sub>9</sub> sequence (designated SIII; see SEQ ID NOS: 10 and 11). IRES activity increased up to 243-fold over background for constructs containing the SII spacer and up to 31-fold for constructs containing the SIII spacer. In all cases, increasing the number of copies of the IRES module resulted in increased IRES activity, although the level of activity was affected differently by the different spacer  
10 sequences. The different activities due to the spacer sequences may be due to an effect on the higher order structure of the mRNA, which may alter the presentation of the IRES modules to the translation machinery. Alternatively, some spacer sequences may introduce nucleotides that contribute to the IRES module, or that may themselves synergize with the IRES modules. However, control experiments using three  
15 different combinations of the spacer sequences alone - (SII/SIII)<sub>5</sub> and (SII/SIII), alternating in position as they do in the experimental constructs - revealed that the spacer sequences did not have any detectable IRES activity (but see Example 1C, below).

20 To confirm that the IRES activity observed with the (Gtx<sub>133-144</sub>)<sub>10</sub>(SI)<sub>9</sub> $\beta$ /RPh construct was independent of the reporter gene, the *Photinus* luciferase gene was replaced with the CAT gene to produce the (Gtx<sub>133-144</sub>)<sub>10</sub>(SI)<sub>9</sub> $\beta$ /RCh construct. Following transfection into N2a cells, CAT activity was determined by thin layer chromatography and liquid scintillation counting. CAT activity in lysates of cells  
25 transfected with this synthetic IRES construct was approximately 103-fold greater than that obtained for a construct containing the EMCV IRES linked to the CAT reporter gene (EMCV/RCh); the activity of a control construct (RCh) was indistinguishable from background. As for the RPh vector, the (Gtx<sub>133-144</sub>)<sub>10</sub>(SI)<sub>9</sub> $\beta$  synthetic IRES also increased *Renilla* expression significantly (about 6.1-fold)  
30 compared to the control RCh construct. The overall ratio of CAT expression to *Renilla* luciferase activity for the synthetic IRES (Gtx<sub>133-144</sub>)<sub>10</sub>(SI)<sub>9</sub> $\beta$ /RCh was about 10.5-fold greater than that of the EMCV IRES. This is a high level of IRES activity,

but is about 6-fold lower than that obtained with constructs containing the *Photinus* luciferase gene as the second cistron. Thus, the reporter cistron may have an influence on IRES activity, as was observed with the EMCV IRES (Kaminski and Jackson, RNA 4:626-638, 1998).

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The activity of some viral IRESes can vary, depending on cell type (Borman et al., *supra*, 1997). Accordingly, the activity of the synthetic (Gtx<sub>133-144</sub>)<sub>10</sub>(SI)<sub>5</sub>β/RCh IRES was examined in 11 different cell lines, including rat C6 glial cells, mouse N2a neuroblastoma cells, human SK-N-SH (SK) neuroblastoma cells, rat B104-1-1 (B104) neuroblastoma cells, mouse NIH-3T3 fibroblasts, human HeLa cervical carcinoma cells, mouse P19 embryonal carcinoma cells, rat NRK normal kidney cells, human K562 chronic myelogenous leukemia cells, mouse AtT-20 pituitary tumor cells, and mouse C2C12 muscle myoblast cells, and compared to that of the EMCV IRES. In all of the cell lines tested, the synthetic IRES was about 3.3-fold (NRK cells) to 63-fold (N2a cells) more active than that of the EMCV IRES.

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To investigate the potential effects of distance between IRES modules, a series of constructs was generated containing 1 (SEQ ID NO: 12) or 2 (SEQ ID NOS: 13 to 15) copies of the Gtx IRES module (SEQ ID NO: 2). Spacing in constructs containing 2 copies of the Gtx IRES module varied from 9 to 54 nucleotides (see SEQ ID NOS: 13 to 15) using multiple copies of the SI spacer sequence, such that all of constructs were identical in size. The IRES activity obtained with 1 copy of the IRES module increased 2.8-fold when a second copy was introduced 9 nucleotides upstream of the first module. This activity decreased to about 2.1-fold and 1.3-fold as the distance between the IRES modules was increased to 27 and 54 nucleotides, respectively. Further experiments revealed that IRES activity continued to increase as the number of multimers was increased to 35, and that multimers of up to 50 Gtx modules (SEQ ID NO: 2), which was the most examined, had IRES activity, though somewhat less than that obtained using 35 copies. These results demonstrate that at least two and at least up to 50 of the Gtx IRES modules (SEQ ID NO: 2) can be operatively linked, and that the IRES activity of the combined modules can be varied by varying the distance between the IRES modules.

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### **C. IRES Activity of Mutant Gtx Modules**

Constructs containing 5 copies of the Gtx IRES module (SEQ ID NO: 2), each separated by the 9 nucleotide SI  $\beta$ -globin leader sequence, or 5 copies of a mutated version of the Gtx IRES module were cloned into the RPh vector, transfected into N2a cells, and examined for luciferase activity. Portions of these constructs are shown in Figure 2 (compare SEQ ID NO: 6, which contains the wild type Gtx IRES module (SEQ ID NO: 2), with SEQ ID NOS: 17 to 19, which contain substitutions in the module; see, also, SEQ ID NOS: 20 to 22, which contain substitutions in the spacer sequence).

Substitution of adenosine residues at the 3' end of SEQ ID NO: 2 increased the IRES activity of the oligonucleotide (compare SEQ ID NO: 6 with SEQ ID NOS: 17 and 19). Changes in the composition of the spacer nucleotides adjacent to the Gtx IRES module either had no effect or also increased IRES activity (compare SEQ ID NO: 6 with SEQ ID NOS: 20 to 22). These results indicate that, when present in a particular context, the Gtx IRES module (SEQ ID NO: 2) provides a defined level of IRES activity, and that additional sequences based on the Gtx IRES module, as well as the context of the module with respect to flanking sequences can provide different levels of IRES activity.

## **EXAMPLE 2**

### **IDENTIFICATION AND CHARACTERIZATION OF IRES ELEMENTS FROM DENDRICTICALLY LOCALIZED RNA MOLECULES**

This example demonstrates that RNA molecules that localize in dendrites contain 5' UTR nucleotide sequences that act as internal ribosome binding sites (IRESes)

#### **A. Vector and Cell Lines**

The 5' untranslated regions (5' UTRs) from the alpha subunit of Cam Kinase II (CamK IIa; SEQ ID NO: 23), dendrin (SEQ ID NO: 24), the activity-regulated cytoskeletal protein (ARC; SEQ ID NO: 25), microtubule-associated protein 2 (MAP2; SEQ ID NO: 26) and neurogranin (RC3; SEQ ID NO: 27) were obtained by

RT-PCR amplification of rat adult hippocampal cDNA. Amplified 5' UTRs corresponded to the transcriptional start sites of the mRNAs and ended immediately 5' to the AUG translation start site. Primers were engineered with restriction endonuclease recognition sites for Eco RI and Nco I, to allow for directional cloning into the dicistronic vectors, RP and RPh, each of which encodes *Renilla* luciferase in the first cistron and *Photinus* luciferase in the second cistron (see Example 1A). All 5' UTRs were verified by sequence analysis.

The monocistronic luciferase vector, PM, was generated by substituting the *Photinus* luciferase gene from the RP construct for the EYFP (enhanced yellow fluorescent protein) open reading frame in the pEYFP-N1 vector, which contains a CMV promoter (Clontech) using the Eco RI and Xba I sites. As an internal control for transfection efficiency, the region of the chloramphenicol acetyl transferase (CAT) reporter gene construct of the pCAT3 control vector, which contains the SV40 promoter, CAT reporter gene and polyadenylation sequences (Promega), was also cloned into the monocistronic vector. The inverted repeat encoding the hairpin structure at the 5' end of RPh was amplified by PCR and inserted into the 5' region of the multiple cloning site in PM to yield the monocistronic hairpin construct PMh.

Monocistronic luciferase constructs were generated from the EYFP-N1 (Clontech) vector by replacing the gene encoding enhanced yellow fluorescent protein (EYFP) with the *Photinus* luciferase gene from the RP constructs using the Eco RI and Xba I restriction sites. The chloramphenicol acetyl transferase (CAT) gene was used as a control for transfection efficiency. A blunt ended Bam HI/Bgl II fragment containing the SV40 promoter, the CAT reporter gene, and polyadenylation sequences from the pCAT3 control vector (Promega) was cloned into the Ase I restriction site of these constructs after filling in the 3' recessed ends using the Klenow fragment of DNA polymerase I.

An EYFP construct (EYFP/NLS) was used to test the ability of the RC3 3' UTR to target mRNA to dendrites. This construct contains a nuclear localization

signal (NLS) and a nuclear matrix binding site (NMBS) attached to the EYFP coding sequence to limit diffusion of EYFP from the cell body to the dendrites.

For *in situ* analysis of IRES activity, a dicistronic fluorophore vector was  
5 constructed that encodes enhanced cyan fluorescent protein (ECFP) as the first cistron and EYFP as the second cistron. Both fluorescent cistrons were modified to contain the mouse ornithine decarboxylase (ODC) destabilizing motif, which shortened the half-lives of these proteins to approximately 4 hr. This motif was obtained from the EGFP-N1 vector (Clontech) as a Bsr GI/Not I fragment. The mRNA expressed from  
10 this construct was targeted to dendrites using the 3' UTR from the RC3 gene.

Rat neural tumor B104 cells, mouse neuroblastoma N2a cells, rat glial tumor C6 cells, mouse NIH 3T3 (3T3) fibroblasts, and mouse LMTK fibroblast cell lines were used for these studies. Cells were prepared and transiently transfected as  
15 previously described (Chappell et al., *supra*, 2000). Primary cultures of dissociated hippocampal neurons were cultured on poly-L-lysine coated chamber slides (Sporns and Jenkinson, *Neuroscience* 90:1057-1073, 1997, which is incorporated herein by reference). Culture media were exchanged twice weekly until neurons had reached maturation (about 21 to 25 days *in vitro*). Hippocampal neurons were transfected  
20 using calcium phosphate (Promega).

#### **B. Reporter Gene Assays**

Cells were prepared 12 to 14 hr prior to transfection by plating approximately 70,000 to 80,000 cells per well in a Falcon 6-well culture cluster. Cells were grown  
25 in DMEM with 10% FBS supplemented with 1% penicillin, streptomycin and glutamine. Dicistronic luciferase vectors (0.5 µg) were co-transfected with 0.2 µg of β-galactosidase reporter vector (CMVβ, Promega) per well using FuGENE 6 transfection reagent according to manufacturer's instructions (Boehringer; Germany). Transfected cells were grown for 22 to 24 hr, then rinsed briefly with PBS and lysed  
30 using 250 µl lysis buffer (Promega). Extracts (20 µl) were assayed for *Renilla* and *Photinus* luciferase activity using the dual luciferase assay kit (Promega).

$\beta$ -galactosidase was measured simultaneously using the fluorescent reporter  $\beta$ -gal assay kit (Molecular Probes; Eugene OR). All constructs were transfected at least three times and individual transfections were performed in triplicate.

5 Monocistronic vector transfections were performed as above, but were not cotransfected with pCMV $\beta$  vector. B104 cells were transfected with monocistronic DNA constructs, then lysed and assayed for *Photinus* luciferase and CAT enzyme activity using single luciferase assay reagents (Promega) and a CAT enzyme assay kit (Promega) according to the manufacturer's instructions. Quantification of CAT  
10 activity was performed using a liquid scintillation counter. In experiments using the 4E-BP1 mutant (see below), either a plasmid expressing the dominant negative form of the eIF4E binding protein 4E-BP1, or an empty expression vector was co-transfected in parallel with the monocistronic constructs (Gingras et al., Genes Devel. 13:1422-1437, 1999, which is incorporated herein by reference).

15

### **C. Northern Blot Analysis**

Total RNA from transfected cell lines was extracted using TRIZOL reagent (Gibco BRL; Gaithersburg MD) and was size separated on a 1.3% agarose/formaldehyde gel. RNA was transferred to nylon membrane using capillary transfer,  
20 baked for 1 hr at 80°C, and prehybridized in 50% formamide, 5X SSC, 50 mM NAHPO<sub>4</sub>, 1% SDS, 5% dextran sulfate, and 200  $\mu$ g/ml polyadenylate for 2 hr at 70°C.

cDNA encoding the full-length *Photinus* luciferase reporter enzyme was  
25 directionally subcloned from the pGL3 (Promega) vector into the PBS SK II (+) vector (Stratagene; La Jolla CA) using Hind III and Xba I restriction sites. The resulting plasmid was linearized with Hind III and used as a template for synthesis by *in vitro* transcription of <sup>32</sup>P-radiolabeled and digoxigenin (DIG)-labeled cRNA probes for northern blot analysis and *in situ* hybridization (Bizon et al., J. Comp.  
30 Neurol. 408:283-298, 1999; Pinkstaff et al., Brain Res. Mol. Brain Res. 55:265-276, 1998, each of which is incorporated herein by reference).



For fluorescent microscopic analysis, transfected neurons were fixed using 4% paraformaldehyde in phosphate buffered solution, placed under cover slips using Slow-Fade reagent (Molecular Probes), and visualized and quantified as described previously (Vanderklish et al., Proc. Natl. Acad. Sci., USA 97:2253-2258, 2000, which is incorporated herein by reference). Images were captured from each fluorescent filter channel for equal time periods and optimized so that the emission from both fluorophores was within a linear range. At least 20 images were quantified for each construct. Experimental data were analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by an individual analysis of experimental versus control data points using a two-tailed Student's t-test. Results were considered significant at  $p < 0.05$ .

**D. Identification of Translational Regulatory Activity In the 5' UTRs of Dendritically Localized mRNAs**

To determine the effect on translation of the full length leader sequences of CamK IIA (SEQ ID NO: 23) dendrin (SEQ ID NO: 24), ARC (SEQ ID NO: 25), MAP2 (SEQ ID NO: 26), and RC3 (SEQ ID NO: 27), the sequences were inserted into the 5' UTR of a monocistronic reporter mRNA (PM), which encodes the *Photinus* luciferase protein, and examined for luciferase activity. The translation of  $\beta$ -globin mRNA is cap-dependent and the 5' UTR of the  $\beta$ -globin mRNA was included in these studies as a control. Monocistronic constructs were transfected into B104 cells. RNA blot analyses of total RNA from transfected B104 cells showed similar intensity of the autoradiographic bands between monocistronic constructs, indicating that the leader sequences do not affect the transcription or the stability of the monocistronic mRNAs.

The *Photinus* luciferase activity of each construct was determined, and the results were normalized for transfection efficiency using CAT activity, which was co-expressed from the PM plasmids. The  $\beta$ -globin 5' UTR had little effect on monocistronic translation. However, mRNA containing the MAP2 (SEQ ID NO: 26), ARC (SEQ ID NO: 25), or CamK IIA (SEQ ID NO: 23) 5' UTR showed enhanced

levels of translation compared to the base vector by up to 2.5-fold. In contrast, monocistronic mRNA containing the dendrin 5' UTR (SEQ ID NO: 24) generated luciferase activity that was approximately 20% less than control values. These results indicate that the 5' UTRs (SEQ ID NOS: 23 to 26) can alter the overall levels of translation of monocistronic mRNAs, and do not alter the stability of the monocistronic mRNAs, which could result in corresponding alterations in luciferase activity.

To determine whether the cap-independent translation occurred by internal initiation, the 5' UTRs from CamK IIA (SEQ ID NO: 23) dendrin (SEQ ID NO: 24), ARC (SEQ ID NO: 25), and MAP2 (SEQ ID NO: 26) were cloned into the intercistronic region of the dicistronic luciferase vector, RP. Constructs were transfected into the neural cell lines (N2a cells, B104 cells, and C6 cells) and assayed for *Renilla* and *Photinus* luciferase activity.

Little or no translation of the second cistron (*Photinus* luciferase) occurs in the base vector (RP) and, consequently, only the first cistron (*Renilla* luciferase) was translated. However, if a sequence that can initiate translation is inserted between the two cistrons, then both the *Renilla* and *Photinus* luciferase cistrons will be translated. The ability of sequences within the intercistronic region to initiate translation of the second cistron is characteristically defined as IRES activity. The results are calculated as a ratio of *Photinus* to *Renilla* luciferase activity and compared to the base vector RP.

Insertion of the 5' UTR of  $\beta$ -globin, translation of which is cap-dependent, resulted in luciferase activity that was comparable to that of the RP vector alone. In contrast, insertion of the EMCV IRES into the intercistronic region resulted in about a 6 to 7-fold increase in translational activity as compared to RP. Insertion of the 5' UTRs of the dendritically localized mRNAs (SEQ ID NOS: 23 to 26) into the intercistronic region also resulted in increased translational activity, particularly with the ARC 5' UTR (SEQ ID NO: 18), which exhibited about 13-fold greater activity than the RP vector alone, and about 2-fold greater than that observed with the EMCV

IRES in C6 cells. The MAP2 (SEQ ID NO: 26),  $\alpha$ -CamK II (SEQ ID NO: 23), and dendrin (SEQ ID NO: 24) 5' UTRs generated approximately 1/3 to 1/2 the translation activity of EMCV, except that the IRES activity generated by the MAP2 5' UTR (SEQ ID NO: 26) was nearly equivalent to the activity of the EMCV IRES in  
5 C6 cells.

The relative levels of IRES activity between the different 5' UTRs generally were similar in all three neural cell lines. However, transfection of C6 cells consistently demonstrated higher ratios than N2a cells or B104 cells for all constructs.  
10 These results demonstrate that the 5' UTRs shown as SEQ ID NOS: 23 to 26, which are from dendritically localized mRNAs, contain sequences sufficient for the internal initiation of translation.

#### **E. Internal Initiation in Non-Neural Cell Lines**

15 To determine whether the 5' UTRs could also function as IRESes in non-neural cell types, the dicistronic constructs were transfected into 3T3 (fibroblast) cells and LMTK (kidney) cells. Each of the dicistronic constructs containing the 5' UTRs from the dendritically localized CamK IIA (SEQ ID NO: 23) dendrin (SEQ ID NO: 24), ARC (SEQ ID NO: 25), and MAP2 (SEQ ID NO: 26) mRNAs were  
20 expressed significantly above control values, demonstrating that IRES activity was conferred by these sequences in both neural and non-neural cell lines. Furthermore, the relative IRES activity generated from the dicistronic mRNAs in non-neural cells was similar to that observed in neural cell lines, with the ARC 5' UTR (SEQ ID NO: 25) being greater than the EMCV IRES, and the MAP2 5' UTR (SEQ ID  
25 NO: 26) being consistently greater than CamK IIA (SEQ ID NO: 23) and dendrin (SEQ ID NO: 24). However, the IRES activity in non-neural cells for dendrin, ARC, and MAP2 5' UTRs was higher than that observed for any of the neural cell lines. These results demonstrate that the 5'UTRs of these dendritically localized mRNAs can internally initiate translation in neural and non-neural cell lines, and that the  
30 overall level of IRES activity varies among cell types.

#### **F. Dicistronic mRNAs Are Not Alternatively Spliced**

To determine if translation of the second cistron was due to alternative splicing of the dicistronic mRNAs resulting in the generation of two monocistronic messages, a northern blot analysis of total RNA from N2a cells transfected with the  
5 CamK IIA (SEQ ID NO: 23) dendrin (SEQ ID NO: 24), ARC (SEQ ID NO: 25), and  
MAP2 (SEQ ID NO: 26) dicistronic constructs was performed. Equivalent amounts  
of total RNA were hybridized with a cRNA probe derived from the full-length  
*Photinus* luciferase open reading frame. Using this probe, any message that is spliced  
is detected as a lower molecular weight fragment. However, no apparent lower  
10 molecular weight fragments were present, and the hybridized cRNA probe detected  
bands of the correct size for all dicistronic messages. This result demonstrates that  
the dicistronic constructs are translated as a single RNA species.

#### **G. Translation of the Second Cistron is Not Due to Re-Initiation or**

##### **Read-Through**

In addition to internal initiation, translation of the second cistron also can be  
due to re-initiation and read-through from the first cistron. To eliminate this  
possibility, the CamK IIA (SEQ ID NO: 23) dendrin (SEQ ID NO: 24), ARC (SEQ  
ID NO: 25), and MAP2 (SEQ ID NO: 26) 5' UTRs were cloned into the RPh vector,  
20 which contains an inverted repeat sequence at the 5' end of the first cistron. The  
inverted repeat forms a stable hairpin loop structure and inhibits cap-dependent  
translation of the first cistron, dramatically reducing the levels of *Renilla* luciferase  
activity.

25 The dicistronic constructs were transfected into B104 cells and assayed for  
luciferase activity. Luciferase activity ratios for each of the four 5' UTRs was  
significantly greater than background RPh ratios, demonstrating that the 5' UTRs  
direct internal initiation of translation of the second cistron even when translation of  
the first cistron is inhibited. The ratio of *Photinus* to *Renilla* for the 5' UTRs from the  
30 dendritically localized mRNAs was elevated as compared to that of sequences lacking  
the hairpin structure, indicating that inhibition of cap-dependent translation can  
enhance cap-independent translation initiated by the 5' UTRs (SEQ ID NOS: 23

to 26). In comparison, the 5' UTR of  $\beta$ -globin was not significantly different from RPh luciferase activity, and the EMCV IRES activity was maintained in the presence of the hairpin. These results indicate that translation of the second cistron of dicistronic mRNAs is due to internal initiation, and not read-through or re-initiation from the first cistron, thus confirming that the 5' UTRs (SEQ ID NOS: 23 to 26) of the dendritically localized mRNA molecules examined act as IRES elements.

#### **H. 5' UTRs Confer Cap-Dependent and Cap-Independent Translation**

To examine whether the CamK IIA, dendrin, ARC, and MAP2 5' UTRs (SEQ ID NOS: 23 to 26, respectively) function through both cap-dependent and cap-independent mechanisms of translation in monocistronic mRNAs, a hairpin was constructed at the 5' end of the monocistronic vector PM. This base vector (PMh) was used to examine the ability of the 5' UTRs to direct internal initiation of translation without the influence of ribosomal cap binding and scanning. The PMh-based constructs were transfected into B104 cells and assayed for both *Photinus* and CAT enzymatic activity.

The construct containing the  $\beta$ -globin 5' UTR exhibited luciferase activity that was slightly below that of the base vector, demonstrating that, as shown previously, the  $\beta$ -globin 5' UTR does not initiate translation internally. All four UTRs from the dendritically localized mRNAs (SEQ ID NOS: 23 to 26) generated luciferase activity above that produced by the base vector. As seen with the other constructs, the ARC 5' UTR (SEQ ID NO: 25) generated the most robust activity. However, the relative levels of luciferase activity produced by these 5' UTRs differed from that seen in monocistronics without hairpins. In particular, the dendrin 5' UTR (SEQ ID NO: 24), which demonstrated the least amount of IRES activity compared to the other 5' UTRs and actually decreased the level of luciferase activity in monocistronic RNA, produced a 3-fold increase in IRES activity in the hairpin-containing monocistronic RNA. Also, the MAP2 5' UTR (SEQ ID NO: 26) was not as robust when placed downstream of the hairpin as it was in the monocistronic mRNA. These results indicate that all four of the 5' UTRs (SEQ ID NOS: 23 to 26) utilize internal initiation of translation to different extents in the monocistronic RNAs.

Several mechanisms can regulate cap-dependent eukaryotic translation, including the modulation of eIF4E binding to the cap structure. To address whether these 5' UTRs could initiate translation without eIF4E binding to the cap, monocistronic 5' UTR constructs were co-transfected with a plasmid expressing a dominant negative form of the 4E-binding protein, 4E-BP1. 4E binding proteins can inhibit cap-dependent translation by sequestering eIF4E, thereby preventing eIF4E from binding the cap structure, and the eIF4E/4E binding protein complex can be dissociated due to phosphorylation of 4E binding protein. Mutation of the phosphorylation sites results in a dominant negative form of the 4E-BP1.

Cotransfected B104 cells were grown for 24 hr and assayed for *Photinus* luciferase activity. Luciferase activity from cells cotransfected with the plasmid encoding the mutant 4E-BP1 plasmid was expressed as a percentage of the luciferase activity in cells cotransfected with a control expression plasmid. Expression of the 4E-BP1 mutant reduced the luciferase activity from the PM and the  $\beta$ -globin monocistronics to 45% and 43%, respectively, of the luciferase activity from cells containing the same 5' UTR (SEQ ID NOS: 23 to 26) constructs transfected with the control expression plasmid. All of the constructs containing the 5' UTRs from the dendritically-localized mRNAs (SEQ ID NOS: 23 to 26) had luciferase activities that were above those of PM and  $\beta$ -globin. This result indicates that translation of these mRNAs occurs by both cap-dependent and -independent mechanisms.

The luciferase activity produced by the ARC (SEQ ID NO: 25) and MAP2 (SEQ ID NO: 26) 5' UTRs was reduced by 38% and 32% of control, respectively, yet remained approximately 45 to 50% higher than PM and  $\beta$ -globin, indicating both cap-dependent and cap-independent contributions to the translation of monocistronic mRNA. However, the CamK IIa 5' UTR (SEQ ID NO: 23), which strongly enhanced the translation of a monocistronic mRNA, was only reduced by about 20% of control when cotransfected with the 4E-BP1 mutant. Interestingly, the dendrin 5' UTR (SEQ ID NO: 24), which had the least IRES activity in dicistronic mRNAs, demonstrated luciferase activity that was only 13% less than control in the presence of 4E-BP1

mutant, indicating that IRES-dependent translation may be a major contributor to its overall levels of translation. These results are consistent with the results obtained using the hairpin constructs, and demonstrate that translation of monocistronic mRNAs containing a 5' UTRs from the four dendritically localized mRNAs (SEQ ID NOS: 23 to 26) occurs by both cap-dependent and cap-independent mechanisms.

### **I. Characterization of RC3 Translational Regulatory Element**

The RC3 5' UTR (SEQ ID NO: 27) was also analyzed in the dual luciferase dicistronic mRNA. Northern blot analysis of the RC3 5' UTR in the RP construct resulted in the appearance of two mRNAs. Removal of the chimeric intron from the RP vector resulted in the production of one mRNA of the correct size predicted for the dicistronic mRNA. The leader sequence of  $\beta$ -globin mRNA as well as the EMCV IRES were cloned into the intercistronic region of this modified intron-less construct (RPi) and were analyzed in transiently transfected B104 cells. The resulting  $\beta$ -globin and EMCV P:R ratios were similar to those generated by the dicistronic mRNAs containing the chimeric intron. The P:R ratios produced from dicistronic mRNAs containing the RC3 5' UTR (SEQ ID NO: 27) was approximately 18-fold higher than RPi and approximately 3-fold higher than that obtained with the EMCV IRES. The P:R ratio obtained with constructs containing a hairpin structure 5' of the first cistron were not significantly different from those obtained with the RP construct. These results indicate that the RC3 leader sequence (SEQ ID NO: 27) also contains an IRES.

The activity of RC3 IRES was examined in primary hippocampal neurons. To analyze protein synthesis in dendrites, a monocistronic fluorophore reporter construct was engineered to target the mRNAs to dendritic processes. Targeting sequences have been identified within the 3' UTRs of several dendritically localized mRNAs (Kiebler and DesGroseillers, *Neuron* 25:19-28, 2000, which is incorporated herein by reference). To determine if the RC3 3' UTR functioned similarly, it was included in the 3' UTR of a monocistronic mRNA encoding EYFP along with a nuclear localization sequence (EYFP/NLS). The inclusion of the RC3 3' UTR resulted in expression of a significant amount of the fluorescent protein in dendrites of

transfected neurons; constructs lacking this 3' UTR expressed the fluorescent protein only in the nucleus. This result suggests that the RC3 3'UTR could target mRNA to the dendrites of transfected neurons.

- 5           *In situ* hybridization confirmed that reporter mRNAs containing the RC3 3' UTR were localized throughout the processes of transfected hippocampal neurons, whereas reporter mRNAs lacking this 3' UTR were confined to the cell body. In addition, co-localization studies using the MAP2 antibody, which specifically stains dendrites, showed that mRNAs containing the RC3 3' UTR occurred within dendrites.
- 10   These results validated the use of the RC3 3' UTR in directing mRNAs to the dendritic compartment and also demonstrated that translation occurs in dendrites.

- To examine whether IRES mediated translation can occur within dendrites, a dicistronic mRNA containing ECFP as the first cistron, EYFP as the second cistron,
- 15   and the RC3 3' UTR was used. To limit diffusion from the site of synthesis and facilitate quantification of *de novo* local protein synthesis, both fluorescent proteins were engineered to contain the mouse ODC destabilizing motif, which shortened the half-lives of these proteins to approximately four hours. As an initial analysis of the dicistronic fluorophore mRNAs, the constructs were tested in transfected N2a cells;
- 20   IRES activities using this construct were similar to those observed using the dual luciferase vector.

- Hippocampal neurons were transfected with dicistronic ECFP/EYFP constructs containing the 5' leader sequences from  $\beta$ -globin and RC3 (SEQ ID
- 25   NO: 27) mRNAs within the intercistronic region. Fluorescence measurements were taken from the cell body as well as from the dendrites. Fluorescent overlay images using a ECFP and EYFP filter were taken from the dendritic fields of two neurons transfected with dicistronic fluorophore constructs containing either the  $\beta$ -globin or RC3 leader sequence in the intercistronic region, respectively. Transfection of
- 30   hippocampal neurons with the dicistronic construct containing the  $\beta$ -globin 5' UTR resulted in mainly cap-dependent ECFP expression (visualized as cyan). However,



the mRNA containing the RC3 leader sequence (SEQ ID NO: 27) demonstrated both cap-dependent translation and IRES-dependent translation (visualized as yellow) generating turquoise. Fluorescence activity of both cistrons was quantified from images and calculated as a yellow:cyan ratio.

5

The results demonstrated that the RC3 leader functioned as an IRES in both the cell body and the dendrites. Distribution of the activity of the RC3 IRES was punctate in the dendrites, indicating that activity was increased near synapses. In addition, the relative activity of the RC3 IRES (SEQ ID NO: 27) was approximately  
10 2-fold more active in the dendrites than in the cell body. A northern blot of these dicistronic fluorophore mRNAs contained a single hybridizing band of the correct molecular weight.

Since many synaptic modifications do not persist when translation is inhibited,  
15 translation has been suggested to have an essential role in mediating changes in the morphology and efficacy of individual synapses in response to synaptic activity. Moreover, it is thought that some synaptic changes result from translation at post-synaptic sites rather than from cell-wide changes in protein synthesis. Such local translation would enable different synapses to be modified independently within the  
20 same cell. Evidence to support the notion of local protein synthesis includes the observations that components of the translation machinery occur in dendrites, that protein synthesis can occur at these cellular processes, and that many dendritically localized mRNAs encode proteins that are integral to synaptic modifications, including structural proteins (ARC, MAP2), growth factors, ionotropic receptors, and  
25 kinases (CamK IIa; see Kuhl and Skehel, Curr. Opin. Neurobiol., 8:600-606, 1998). These observations suggest that translation in dendrites is used to rapidly and locally synthesize some of the proteins required to strengthen active synapses.

As disclosed herein, the leader sequences of five dendritically localized  
30 mRNAs (SEQ ID NOS: 23 to 27) contain IRES activity, which mediates cap-independent translation. In addition, translation mediated by the RC3 5' UTR (SEQ ID NO: 27) occurred by both cap-dependent and cap-independent mechanisms within

dendrites of cultured hippocampal neurons. Furthermore, translation by the cap-independent mechanism was relatively more efficient in the dendrite than in the cell body.

5           These 5' UTR sequences having IRES activity can serve to ensure or regulate translation of specific messages under varying conditions and can be involved in effecting synaptic plasticity. The identification of an IRES in the 5' UTR of the mRNA encoding the alpha subunit of Cam Kinase II is consistent with results showing increased translation of this mRNA following treatment of developing  
10       synapses with NMDA receptor agonists, even though total protein synthesis was impaired (Scheetz et al., Nat. Neurosci. 3:211-216, 2000). It has also been reported that the 5' UTR of the mRNA encoding Pim kinase, a protein that has recently been implicated in the stabilization of LTP also contains an IRES (Johannes et al., Proc. Natl. Acad. Sci., USA 96:13118-13123, 1999).

15           Inasmuch as ribosomes and initiation factors are present at low concentrations in dendrites, IRESes can be used by dendritically localized mRNAs to increase their ability to recruit the translation machinery within this cellular compartment. Indeed, IRESes appear to be used by some mRNAs when cap-dependent translation is  
20       reduced or blocked. For instance, overall levels of translation are reduced during apoptosis and during various types of cellular stress such as ischemia (Sheikh and Fornace, Oncogene 18:6121-612, 1999) and in these situations, IRES-mediated translation continues (Holcik et al., Nat. Cell. Biol. 1:190-192, 1999; Stein et al., Mol. Cell. Biol. 18:3112-3119, 1998; Stoneley et al., Mol. Cell. Biol. 20:1162-1169, 2000).  
25       In addition, it has been reported that synaptic activity leads to an increase in protein synthesis in or near the active synapses, an increase that could saturate the cap-dependent translation machinery in dendrites. Thus, internal initiation can provide a means to initiate translation of dendritically localized messages in response to synaptic stimuli.

30

### EXAMPLE 3

#### IDENTIFICATION OF TRANSLATIONAL REGULATORY ELEMENTS THAT ARE ACTIVE IN GROWING YEAST CELLS

This example demonstrates that translational regulatory elements are present  
5 in mRNAs encoded in yeast cells, and that isolated elements of the yeast mRNA  
sequences have translational regulatory activity in vegetatively growing yeast cells.

In higher eukaryotes, translation of some mRNAs occurs by internal initiation.  
It is not known, however, whether this mechanism is used to initiate the translation of  
10 any yeast mRNAs. As disclosed herein, naturally occurring nucleotide sequences that  
function as IRES elements within the 5' leader sequences of *Saccharomyces*  
*cerevisiae* YAP1 and p150 mRNAs were identified. When tested in the 5' UTRs of  
monocistronic reporter genes, both leader sequences enhanced translation efficiency  
in vegetatively growing yeast cells. Moreover, when tested in the intercistronic  
15 region of dicistronic mRNAs, both sequences exhibited IRES activity that functioned  
in living yeast cells. The activity of the p150 leader was much greater than that of the  
YAP1 leader. Further analyses of the p150 IRES revealed several non-overlapping  
segments that independently mediated internal initiation. These results demonstrate  
that the p150 IRES has a modular structure similar to IRES elements contained within  
20 some cellular mRNAs of higher eukaryotes. Both YAP1 and p150 leaders contained  
several complementary sequence matches to yeast 18S rRNA.

The plasmid pMyr (Stratagene) was used as backbone for both dicistronic and  
monocistronic constructs. An adaptor containing restriction sites Hind III, Pst I,  
25 Nhe I, Eco RI, Nco I, and Xba I was introduced into the pMyr vector immediately  
downstream of the GAL1 promoter, using Hind III and Xba I as cloning sites. The  
PstI and XbaI sites were used as cloning sites for a fragment from the RPh dicistronic  
reporter vector (see Example 1A). The resulting construct, pMyr-RP, encodes a  
dicistronic mRNA that encodes *Renilla* (sea pansy) and *Photinus* (firefly) luciferase  
30 proteins as the first (upstream) and second (downstream) cistrons, respectively. These  
cloning steps resulted in a 5' UTR that differs slightly from that in the RP mRNA

described previously (Example 1A). The CYC1 terminator sequence contained within pMyr-1 vector provides signals for termination of transcription and polyadenylation.

5           The p150, YAP1, and CLN3 leader sequences were PCR amplified using yeast genomic DNA as a template. These leader sequences were cloned into the intercistronic region of the pMyr-RP vector using Eco RI and Nco I restriction sites that were introduced at the 5' and 3' ends of the leader sequences to generate constructs designated as pMyr-p150/RP, pMyr-YAP1/RP, and pMyr-CLN3/RP. A  
10   hairpin structure with a predicted stability of  $-50 \text{ kcal mol}^{-1}$  (Stoneley et al., *supra*, 1998) was introduced into the 5' UTR of the dicistronic constructs to generate pMyr-p150/RPh, pMyr-YAP1/RPh, and pMyr-CLN3/RPh. Deletions and fragments of the p150 leader were generated by PCR amplification of the p150 sequence, again using Eco RI and Nco I as cloning sites.

15           Monocistronic constructs containing the *Photinus* luciferase gene were generated in the modified pMyr vector. The *Photinus* luciferase gene was obtained from the pGL3 control vector (Promega) as an Nco I/Xba I fragment and cloned using these same sites to generate construct pMyr/P. The leader sequences from YAP1,  
20   p150, and CLN3 mRNAs, as well as the hairpin structure were cloned into the pMyr/P vector using the same restriction sites used for the dicistronic constructs. Constructs containing the CAT gene were cloned into the pGAD10 vector (Clontech). The pGAD10 vector was digested with Hind III and an adaptor containing restriction sites Hind III, Pst I, Nhe I, Eco RI, Nco I, and Xba I was introduced into this site, which is  
25   immediately downstream of the ADH promoter. The CAT gene was obtained from the pCAT3 control vector (Promega) and cloned into the modified pGAD10 vector using Nco I and Xba I restriction sites. The p150 leader sequence was introduced into this vector as an Eco RI/Nco I fragment to generate the construct designated  
30   p150/CAT. The hairpin structure described above was introduced 5' of this leader sequence to generate the construct designated p150/CATh.

The yeast strain EGY48 (*MAT $\alpha$* , *his3*, *trp1*, *ura3*, *LexA<sub>op(X6)</sub>-LEU2*; Clontech) was used throughout the study. Yeast strains harboring the pMyr based plasmids were grown overnight in 4 ml synthetic defined medium (SD) with uracil and glucose. The following morning, cells were harvested, washed with 4 ml H<sub>2</sub>O, and grown for  
5 3 hr in 4 ml SD medium without uracil with the addition of 2% galactose and 1% raffinose. Cells harboring the pGAD10 constructs did not require induction and were cultured in 4 ml SD/Ura glucose medium overnight. Cells were lysed with 1x lysis buffer (diluted freshly from 5x stock; Promega) in tubes with glass beads. Tubes were vortexed twice for 30 sec and recovered in a microfuge spun at top speed for  
10 3 min at 4°C. The supernatant was recovered and 20  $\mu$ l of the lysate was used to assay luciferase activities using the dual reporter assay system (Promega). CAT activity was measured using N-butyl CoA according to technical bulletin no. 84 (Promega).

15 RNA was isolated from 4 ml cell culture samples. Cells were pelleted, washed with water, and resuspended in 400  $\mu$ l of TES buffer (100 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% SDS). RNA was extracted using preheated phenol (65°C); the mixture was vortexed for 1 min and incubated at 65°C for one hr. Samples were put on ice for 5 min, then centrifuged at 15,000 rpm for 5 min and the  
20 top aqueous phase was collected, re-extracted with phenol once and chloroform once. RNA was precipitated with isopropanol, the precipitate was washed with 70% ethanol, dried and dissolved in water. RNA samples were separated by gel electrophoresis using 1% formaldehyde/agarose gels and transferred to Nytran SuperCharge nylon membrane (Schleicher & Schuell). The blots were probed with  
25 full-length fire-fly luciferase RNA antisense probe that was labeled with <sup>32</sup>P.

#### A. Analysis of YAP1 5' UTR

The 164 nucleotide YAP1 leader sequence (SEQ ID NO: 28) was examined for translational regulatory activity in the 5' UTR of a firefly (*Photinus*) luciferase  
30 reporter mRNA (YAP1/P). Cells were transformed with constructs expressing the parent *Photinus* (-/P) mRNA, the YAP1/P mRNA, or the 364 nucleotide 5' leader of

the CLN3/P mRNA as a spacer control. Transcription of these monocistronic mRNAs was under control of the GAL1 promoter; mRNA expression was induced with galactose, cells were lysed after 3 hr, and luciferase activities determined and normalized to *Photinus* luciferase mRNA levels. Translation efficiency of the YAP1/P mRNA was approximately 10-fold greater than that of either the control -/P or CLN3/P mRNAs. This result indicates that the YAP1 5' UTR has translational enhancing activity.

To determine whether the translation mediated by the YAP1 transcribed leader sequence has a cap-independent component, it was tested in a dicistronic mRNA for its ability to mediate internal initiation. The leader sequence of YAP1 mRNA was placed in the intercistronic region of a dual luciferase dicistronic mRNA and examined for IRES activity. In these mRNA transcripts, the upstream cistron encodes *Renilla* (sea pansy) luciferase and the downstream cistron encodes *Photinus* luciferase. Cells were transformed with constructs encoding the parent RP mRNA, or with constructs containing the YAP1 or CLN3 leaders in the intercistronic region of the RP mRNA. The YAP1 leader sequence enhanced the translation of the downstream *Photinus* luciferase cistron approximately 5-fold relative to that of the RP mRNA. In contrast, the CLN3 leader had almost no effect on the expression of the second cistron relative to that of the RP mRNA.

Hairpin structures were inserted in the discistronic constructs upstream of the *Renilla* luciferase gene to block scanning and, thereby, reduce the translation of this reporter molecule. The hairpin structures blocked *Renilla* luciferase expression by greater than 90%. Nevertheless, the YAP1 leader permitted translation of the *Photinus* luciferase gene, even when translation of the *Renilla* luciferase gene was blocked. This result demonstrates that the YAP1 leader did not increase expression of the second cistron by reinitiation or leaky scanning.

To exclude the possibility that enhanced expression of the downstream cistron was from shorter, monocistronic mRNAs generated by mechanisms such as RNA fragmentation or an unusual splicing event, RNA was isolated from transformed cells

and analyzed by northern blot analysis using a probe to the downstream *Photinus* luciferase gene. The results demonstrated that the dicistronic mRNAs were intact. Thus, translation of the second cistron was not due to initiation via shorter transcripts. Together, these results demonstrate that the YAP1 5' UTR comprises a nucleotide  
5 sequence that has IRES activity and that has translational enhancing activity.

#### **B. Analysis of p150 5' UTR**

The yeast p150 5' UTR also was examined for translational regulatory activity. The 5' leader of the mRNA encoding the p150 protein was determined by primer  
10 extension analysis to contain 508 nucleotides (SEQ ID NO: 29 see, also, Goyer et al., Mol. Cell. Biol. 13:4860-4874, 1993, which is incorporated herein by reference). This sequence contains 11 open reading frames (ORFs) and does not appear to contain or be part of an intron (Costanzo et al., Nucl. Acids Res. 28:73-76, 2000, which is incorporated herein by reference), consistent with the observation that only  
15 4% of yeast genes contain introns, 90% of which encode ribosomal proteins. The presence of the upstream ORFs in the p150 leader might be expected to inhibit translation by a scanning mechanism.

The p150 sequence was tested in the 5' UTR of a monocistronic reporter  
20 mRNA. Constructs containing this sequence enhanced the translation efficiency of the reporter gene up to 10-fold. However, the analysis was complicated by the appearance of a second band approximately 1 kb, which may be a partial degradation product of the luciferase mRNA; this RNA was too short to encode a functional *Photinus* luciferase protein. Accordingly, the p150 leader was tested in the 5' UTR of  
25 the CAT reporter gene to further evaluate whether it was functioning as a translational enhancer. The results obtained using the CAT reporter construct were similar to those obtained with the *Photinus* luciferase reporter gene; the p150 leader sequence enhanced the translation efficiency of the CAT reporter gene 9-fold.

30 To determine whether any translation mediated by the p150 5' leader was cap-independent, a hairpin structure was inserted at the 5' end of this construct. Although

the hairpin structure inhibited translation of a control CAT mRNA by greater than 90%, translation mediated by the p150 leader sequence was not inhibited but, instead, was enhanced by approximately 3-fold. The CAT mRNA levels did not appear to be affected. These results demonstrate that the translation mediated by this leader  
5 sequence is cap-independent.

To confirm that translation was cap-independent, the p150 leader was tested in the intercistronic region of the dual luciferase RP dicistronic mRNA. In this location, the p150 leader functioned as a potent IRES, enhancing translation of the downstream  
10 *Photinus* luciferase cistron approximately 200-fold relative to that of the RP parent vector. This increase in *Photinus* luciferase activity in the p150/RP mRNA resulted in *Photinus* luciferase protein levels that were approximately twice those of *Renilla* protein levels.

15 Blocking the translation of the upstream *Renilla* luciferase gene with a hairpin structure resulted in an even greater enhancement of the *Photinus:Renilla* luciferase ratio, indicating that the translation facilitated by this sequence was not dependent on the translation of the upstream *Renilla* luciferase cistron. As with the findings with YAP1, the enhanced expression of the downstream cistron was not associated with  
20 RNA fragmentation or unusual splicing events.

The p150 leader sequence was sequentially deleted from the 5' end and fragmented into shorter segments, including fragments consisting of nucleotides 100 to 508, 160 to 508, 250 to 508, 375 to 508, 429 to 508, 481 to 508, 250 to 390, and  
25 1 to 250 of SEQ ID NO: 29, each of which was tested for IRES activity. Most of the IRES activity was associated with nucleotides 160 to 508 of SEQ ID NO: 29. However, all of the fragments examined demonstrated some level of IRES activity. Furthermore, deletion of nucleotides 1 to 100 or nucleotides 100 to 160 of SEQ ID NO: 29 increased translation by internal initiation, indicating that this 160 nucleotide  
30 region contains translational inhibitory sequences, which can inhibit IRES activity. The leader sequence in construct p150(250-508) corresponds to that of a shorter leader sequence that occurs naturally (Goyer et al., *supra*, 1993). This shorter leader



sequence has a level of IRES activity that is similar to that of the entire 508 nucleotide leader (SEQ ID NO: 29).

It was previously noted that many eukaryotic mRNAs contain short  
5 complementary sequence matches to 18S rRNA, raising the possibility that ribosome recruitment at some cellular IRESes might occur by base pairing between mRNA and 18S rRNA (Chappell et al., *supra*, 2000; Mauro and Edelman, *supra*, 1997; Hu et al., *supra*, 1999; Tranque et al., *supra*, 1998). Comparison of the YAP1 and p150 leader sequences to yeast 18S rRNA identified two (SEQ ID NOS: 30 and 32) and four (SE  
10 ID NOS: 34, 36, 38 and 40) complementary sequence matches, respectively, which contained stretches of up to 10 nucleotides of perfect complementarity (Figure 3). In addition, two of the matches are part of more extensive complementary matches of up to 25 nucleotides with 84% complementarity. The complementary match at nucleotides 130 to 142 of the p150 IRES (SEQ ID NO: 34) is correlated with a  
15 60 nucleotide segment of the p150 5' UTR that can inhibit IRES activity. Another complementary match of the p150 IRES at nucleotides 165 to 183 (SEQ ID NO: 36) is correlated with a 90 nucleotide segment of the IRES that contributes to internal initiation. Two other complementary matches of the p150 IRES at nucleotides 423 to 437 (SEQ ID NO: 38) and nucleotides 437 to 461 (SEQ ID NO: 40) are partially or  
20 fully contained within a 52 nucleotide segment with IRES activity.

It was previously suggested that the yeast translation machinery may be capable of mediating internal initiation (Iizuka et al., Mol. Cell. Biol. 14:7322-7330, 1994; Paz et al., J. Biol. Chem. 274:21741-21745, 1999, each of which is incorporated  
25 herein by reference). The present results demonstrate unequivocally that yeast IRES sequences contained within the YAP1 and p150 leader sequences are functional in vegetatively growing cells. In addition, numerous sequences sharing complementarity with yeast 18S rRNA were identified within both leader sequences (Figure 3). Many other mRNAs and cellular IRESes contain similar features, and the  
30 complementary sequence matches to 18S rRNA can function as cis-acting sequences that affect translation (see, for example, Chappell et al., *supra*, 2000). In the case of the Gtx IRES module (SEQ ID NO: 2), this segment is 100% complementary to a

sequence of 18S rRNA. Recruitment of ribosomes at this site appeared to involve base pairing to 18S rRNA within 40S ribosomal subunits. These results indicate that recruitment of ribosomes at some cellular IRES element, including the yeast YAP1 and p150 IRESes, can occur directly due to base pairing to rRNA, a mechanism  
5 consistent with the modular nature of these cellular IRES elements.

The leader sequence of the YAP1 mRNA contained an IRES element that contributed to the efficient translation of this mRNA. Sequence features of this leader previously have been shown to affect translation and mRNA stability (Vilela et al.,  
10 Nucl. Acids Res. 26:1150-1159, 1998; Ruiz-Echevarria and Peltz, Cell 101:741-751, 2000, each of which is incorporated herein by reference). One of these features, a short upstream open reading frame (uORF) did not inhibit translation of the main ORF, even though it was recognized by a large fraction of the scanning ribosome. Inasmuch as uORFs generally inhibit the translation of downstream cistrons, these  
15 results indicated that reinitiation and leaky scanning were also involved in the efficient translation of the YAP1 mRNA.

The p150 IRES element was particularly active. Although most of the IRES activity was localized to nucleotides 160 to 508 (SEQ ID NO: 29), the IRES  
20 boundaries were not distinct. Moreover, several non-overlapping segments functioned independently, suggesting that this IRES has a modular composition. Using methods as described in Example 1, which identified the IRES module (SEQ ID NO: 2) present in the Gtx homeodomain protein, IRES modules in the p150  
5' UTR similarly can be isolated.

25

The notion that short nucleotide sequences can recruit the translation machinery is not consistent with the proposal that higher order RNA conformations are uniformly important for the activity of some cellular IRESes. Indeed, the results obtained from deletion and fragment analyses of IRESes contained within other  
30 mammalian and insect cellular mRNAs indicates that many of these IRESes may also be modular (see, for example, Yang and Sarnow, Nucl. Acids Res. 25:2800-2807, 1997; Sella et al., Mol. Cell Biol. 19:5429-5440, 1999). The modular composition of

cellular IRESes contrasts with those of viruses. For example, in picornaviruses, the IRESes comprise several hundred nucleotides and contain RNA conformations that appear to be highly conserved and that are important for activity.

- 5           It is not known how widely internal initiation is used by yeast or higher eukaryotic mRNAs. The identification of numerous insect and mammalian IRESes may reflect a more extensive use of this mechanism in higher eukaryotes, or it may reflect incidental bias that has resulted in the evaluation of many more mRNAs from insects and mammals than from yeast. Some mammalian IRESes do not function in  
10   living yeast. In the case of poliovirus, the inactivity of its IRES in *S. cerevisiae* reflects a specific blockage that occurs via a short inhibitory RNA. The inactivity of some mammalian IRESes in yeast may also reflect trans-acting factor requirements that are not provided by yeast cells or differences related to the ability of a sequence to bind a component of the translation machinery that is not identical to that in yeast.  
15   For example, p150 is the yeast homologue of mammalian translation initiation factor eIF4G, but the two are not functionally interchangeable.

- In higher eukaryotes, IRESes are used by some mRNAs during the G2/M phase of the cell cycle and under conditions that reduce cap-dependent translation, as  
20   seen, for example, during different types of stress. In yeast, internal initiation may also be used to facilitate the translation of essential genes under similar conditions, including during periods of nutritional deficiency. It may be significant that IRESes were identified within the YAP1 and p150 leader sequences given that overexpression of YAP1 confers general resistance to many compounds, and that expression of p150  
25   when cap-dependent translation is reduced can allow translation of other mRNAs under adverse conditions.

- The identification of yeast IRESes that function in vegetatively growing cells suggests that yeast and higher eukaryotes use similar mechanisms to initiate  
30   translation. The analysis of these mechanisms should be facilitated in yeast, since many strains of yeast having mutations in genes involved in translation are available. The ability to easily manipulate this organism genetically can facilitate the

identification of specific factors involved in internal initiation and confirmation that base pairing between certain IRES sequences and 18S rRNA is important for recruitment of ribosomes at these sites. In addition to these scientific interests, the identification of yeast IRESes that function as translational enhancers in  
5 monocistronic mRNAs also provides numerous applications for bioengineering.

#### EXAMPLE 4

##### **IDENTIFICATION AND CHARACTERIZATION OF SYNTHETIC IRES ELEMENTS**

10 This example demonstrates that translational regulatory elements, including IRES elements, can be identified by screening libraries of random oligonucleotides.

##### **A. Retroviral Vector**

Retroviruses are extremely useful tools to deliver genes into eukaryotic cells  
15 both in culture and in whole animals. Currently, however, most retroviral vectors are not tailored for tissue specific or developmental stage specific delivery of genes. Thus, a benefit of screening a retroviral library for functional synthetic translational regulatory elements as disclosed herein is the potential to create novel retroviruses with exquisite target specificity. Such vectors can be extremely useful for generating  
20 cell lines or transgenic animals for diagnostic screening procedures and drug development. In addition, such vectors can be useful for gene therapy in humans.

A retrovirus is a single stranded RNA virus that infects a cell and integrates into the genome of a cell by copying itself into a double stranded DNA molecule by  
25 reverse transcription. The integrated retrovirus genome is referred to as a provirus. Retroviruses have a two stage life cycle, existing in both an RNA and a DNA form. The RNA form of the virus is packaged into an infectious particle that is coated with a glycoprotein that is recognized by receptors on the host cell. This interaction promotes a receptor mediated internalization event, resulting in exceptionally efficient  
30 delivery of the viral genome into the cell. After transport to the cell nucleus and uncoating, the RNA genome is reverse transcribed into a DNA form (a provirus). During the reverse transcription process, the provirus integrates into the host cell

genome. Retroviruses do not integrate in a completely random fashion, but instead have a distinct preference for integration into regions of the genome that are transcriptionally competent. This characteristic reduces the likelihood that the provirus will be silenced by integration into a transcriptionally repressive domain.

5

In a recombinant retrovirus, the entire coding region of the virus is removed and replaced with a transgene. This replacement is done by standard molecular biological techniques using a proviral version of the virus that is propagated as a bacterial plasmid (a pro-retroviral vector). However, other sequences in the retrovirus genome are required for the functions of viral transcription and packaging, including genes encoding the viral gag and pol proteins, and the viral glycoprotein coat. While such sequences can be removed from the pro-retroviral plasmid, they must be provided in trans, for example, on other plasmids that are introduced into the host cell via cellular transfection in order to obtain a fully functional recombinant virus.

Alternatively, these helper functions can be designed to be integrated into the cellular genome of the viral packaging cell line.

Retroviruses have two viral promoters called long terminal repeats (LTRs), one located at each end of the viral genome. The upstream LTR is responsible for promoting transcription of the DNA provirus into the RNA form. The downstream LTR is not used for transcription during the RNA phase of the life cycle. However, during reverse transcription of the RNA into the DNA provirus, the downstream LTR provides a template for the replication of the upstream LTR. Thus, native retroviruses contain identical sequences in their upstream and downstream LTRs.

25

The recombinant retroviral vector designed for the IRES selection procedure was designated MESVR/EGFP/ECFP/RSVPro (SEQ ID NO: 16; see, also, Figure 4). This vector was based on the MESV/IRESneo (Owens et al., *Cancer Res.*, 58:2020-2028 (1998); Mooslehner et al, *J. Virol.*, 64:3056-3058, 1990; Rohdewohld et al, *J. Virol.*, 61:336-343, 1987, each of which is incorporated herein by reference). MESV is a C-type retrovirus that was modified to remove sequences that are necessary for independent replication. As such, the virus can only replicate with the assistance of

30

helper genes that encode the proteins required for viral genome packaging and insertion into the host genome.

Features of the MESVR/EGFP/ECFP/RSVPro vector include that 1) a  
5 multiple cloning site was introduced into the downstream LTR for insertion of the  
exogenous sequences that can regulate transcriptional activity of a transgene encoded  
by the recombinant retrovirus, and the endogenous viral core promoter was replaced  
with a strong basal promoter to potentiate transcription promoting activity of inserted  
sequences; 2) a mutated EGFP encoding sequence followed by a multiple cloning site  
10 to allow insertion of elements to be tested sequences and a sequence encoding ECFP  
to allow assay of translational activity on a single cell basis was introduced;  
3) enhancer elements in the upstream LTR were replaced with those from RSV to  
drive higher levels of RNA genome production in the packaging cells; and 4) an SV40  
origin of replication was inserted in order to increase the copy number of the  
15 retroviral plasmids in the packaging cells. The EGFP and ECFP reporter genes are  
expressed as a single transcript, in which the mRNAs are linked by a spacer sequence,  
which can contain an oligonucleotide to be examined for IRES activity. Expression  
of both reporter genes is controlled by a strong RSV promoter to ensure efficient  
transcription of the RNA viral genome and, therefore, a high viral titer. The multiple  
20 cloning site between the EGFP and ECFP coding sequences facilitates the insertion of  
an oligonucleotide to be examined for translational activity.

The MESVR/EGFP/ECFP/RSVPro(ori) retroviral vector contains 6096 base  
pairs (SEQ ID NO: 16). Following insertion of the oligonucleotides to generate a  
25 library of proviruses, each containing a unique potential translational regulatory  
element in the intercistronic region of the dicistronic reporter cassette, the proviral  
vector library was transfected into mammalian cells together with helper plasmids  
required for viral production including a plasmid that encodes the group antigen (gag)  
and the integrase enzyme (pol) that is packaged with the RNA genome as well as a  
30 plasmid that encodes the vesicular stomatitis virus coat glycoprotein coat (VSV-G).  
The DNA provirus is transcribed to generate the RNA form, which is packaged into  
an infectious viral particle. The viral particle is coated with a glycoprotein that is

recognized by receptors on the host cell leading to receptor-mediated internalization. After entry into the cell nucleus, the RNA genome is reverse transcribed into the DNA form which is stably integrated into the host cell genome.

5           The viral packaging protocol involved a triple transfection into COS1 cells of a library containing pro-retroviral vectors that harbor the putative promoter elements together with the two separate plasmids that encode the gag/pol and VSV-G proteins, respectively. Cellular transcription machinery is used to generate the viral RNA strands that are packaged into viral particles and subsequently bud from the cell  
10           membrane. These viral particles can infect a naive cell as described above. The viral library is fully representative of the original vector library because all viral RNAs were transcribed from the same strong promoter. In contrast, each integrated DNA version of the virus contains a different oligonucleotide sequence (or combination of sequences) in the intercistronic region, which can effect translation of the second  
15           reporter molecule and selection for which indicates IRES activity of the oligonucleotide.

          Packaging of the proviral vector library was achieved by cotransfection of the proviral DNA into COS1 cells together with the packaging genes, which are  
20           contained on two separate helper plasmids, pCMV-GP(sal) and pMD.G. The pCMV-GP(sal) plasmid has a cytomegalovirus promoter (pCMV) driving the genes that encode the group antigen (gag) and reverse transcriptase enzyme (pol) from the Moloney murine leukemia virus (MMLV). The pMD.G plasmid encodes the vesicular stomatitis virus G glycoprotein (Naldini et al., Science 272(5259):263-267,  
25           1996, which is incorporated herein by reference). These two plasmids were cotransfected into COS1 cells along with the library of recombinant retroviral vectors containing putative promoter elements in order to generate a library of retroviruses.

#### **B. Selection of Synthetic Oligonucleotides Having IRES Activity**

30           The disclosed synthetic IRES methodology provides a means for selecting functional IRES elements. The IRES selection method allows the parallel screening of millions of random oligonucleotide elements or combinations of elements for

activity in mammalian cells. Selection of synthetic IRES elements in mammalian cells is facilitated if 1) each cell receives a single unique cassette to avoid selection of inactive elements that are fortuitously present in the same cell as an active element; 2) the synthetic IRES is shielded from the effects of genomic sequences that may activate or repress translation; 3) the delivery system is efficient so that a complex library can be readily screened; and 4) the selection process is stringent and is based on a reporter gene assay that is highly sensitive and faithfully reports the activity of the IRES elements.

10           A library of oligonucleotides was ligated immediately upstream of the second nucleotide sequence of a dicistronic reporter cassette comprising two reporter genes by insertion into a cloning site in the intercistronic spacer sequence. The exemplified reporter cassette (see below) contained nucleotide sequences encoding enhanced green fluorescent protein (EGFP) and enhanced cyan fluorescent protein (ECFP),  
15           which were arranged in a dicistronic construct that allows two separate gene products to be made from a single mRNA that is driven by a single promoter. After infection of cells with the retroviral IRES element library and integration into the genome, each IRES was scored for its translational activity by examining the activity of the ECFP reporter gene relative that of EGFP. After 2 to 3 days of infection, uninfected cells  
20           were selected by FACS to obtain cells expressing both EGFP and ECFP; the level of ECFP expression in each cell reflected the strength of an individual synthetic IRES element cassette, such that highly fluorescent cells are likely to contain highly active IRES elements. After multiple rounds of selection, the IRES sequences were amplified from the cellular genome by PCR and sequenced using an automated DNA  
25           sequencer to determine the identity of each of the synthetic IRES elements. The activity of each selected IRES element was confirmed by amplifying the entire IRES element, inserting the amplified element into a dicistronic luciferase reporter vector, and screening for the second luciferase reporter protein under translational control of the inserted IRES. This method allowed the testing of the regulatory cassette in a  
30           different reporter system, which was more amenable to quantitation of IRES activity levels.



Except as indicated, methods were performed essentially as described in Examples 1 to 3. A pool of random 18mers, flanked on either side by two different invariant sequences each 6 base pairs in length, was prepared and inserted into the Mlu I site in the intercistronic spacer of MESV/EGFP/ECFP/RSVPro (see Figure 4).

5 A library of recombinant retroviruses was made by transiently transfecting COS1 cells together with plasmids encoding the MLV gag/pol genes and the VSV-G glycoprotein gene. The library was introduced into B104 cells, then 48 hr later, the cells were subjected to FACS (see below) and cells expressing high levels of EGFP and ECFP were collected. The selected cells were replated, then sorted again for

10 EGFP and ECFP expression. Genomic DNA was extracted from the twice-selected cells, and the 18mers were isolated by PCR using primers complementary to the sequences flanking the Mlu I cloning site in the vector.

IRES activity of the PCR amplified sequences was confirmed by cloning the

15 fragments into the intercistronic region of the dicistronic reporter vector, RPh (Chappell et al., Proc. Natl. Acad. Sci., USA 97:1536-1541, 2000, which is incorporated herein by reference). Individual plasmid clones were transfected into B104 cells and the luciferase activities of the first cistron (*Renilla* luciferase) and the second cistron (*Photinus* luciferase) were assayed. For a given plasmid clone

20 containing a particular 18mer sequence, an increase in the translation of the second cistron relative to the first cistron and normalized to the empty vector indicated that the 18mer functioned as an IRES element.

Oligonucleotides selected using this method and having IRES activity are

25 shown as SEQ ID NOS: 42 to 46, where the first six nucleotides and last six nucleotides in each of the sequences are the invariant sequences flanking the randomly generated 18 nucleotides. These results demonstrate that synthetic IRES elements can be isolated by screening a library of random oligonucleotides using the disclosed methods.

### C. Selection of Additional IRES Elements

To confirm the validity of the retroviral screening method, and to identify other short sequences with properties similar to those of the Gtx IRES module (SEQ ID NO: 2), B104 cells were infected with two retroviral libraries that contained

5 random sequences of 9 or 18 nucleotides in the intercistronic region. Cells expressing both cistrons were sorted and sequences recovered from selected cells were examined for IRES activity using a dual luciferase dicistronic mRNA. Two novel IRES

10 elements were identified, each of which contained a sequence with complementarity to 18S rRNA. When multiple copies of either element were linked together, IRES activities were dramatically enhanced. Moreover, the synthetic IRESes were

differentially active in various cell types. The similarity of these properties to those of the Gtx IRES module (SEQ ID NO: 2) provides confirmatory evidence that short nucleotide sequences can function as translational regulatory elements.

15 Two retroviral libraries were generated. In the first library, an oligonucleotide containing 18 random nucleotides (N)<sub>18</sub> was cloned into the Mlu I site of the polylinker. The sequence of this oligonucleotide is: acgcgtgatcca(N)<sub>18</sub>cgagcgacgcgt (SEQ ID NO: 47; see Edelman et al., *Proc. Natl. Acad. Sci., USA* 97:3038-3043, 2000, which is incorporated herein by reference). In the second library, an

20 oligonucleotide containing two segments of 9 random nucleotides (N)<sub>9</sub> was cloned into the Pac I and Mlu I sites of the polylinker. The sequence of this oligonucleotide was ttaattaagaattcttctgacat(a)<sub>9</sub>ttctgacat(a)<sub>9</sub>ttctgacat(a)<sub>9</sub>(N)<sub>9</sub>(a)<sub>9</sub>(N')<sub>9</sub>(a)<sub>9</sub> - gactcacaacccagaaacagacatacgcgt (SEQ ID NO: 48), where N and N' are different

25 random nucleotide sequences. The design of this oligonucleotide was based on the oligonucleotide (S<sub>III</sub>/S<sub>II</sub>)<sub>5</sub>β (see Example 1). This oligonucleotide did not have IRES activity and was used as a spacer control. The first library consisted of about

2.5 x 10<sup>5</sup> bacterial clones and the second consisted of about 1.5 x 10<sup>5</sup> bacterial clones. As such; each library represented only a small fraction of the potential sequence complexity of the random oligonucleotides (about 6.9 x 10<sup>10</sup>).

The retroviral libraries were packaged in COS1 cells. Subconfluent cells were triply transfected using the FuGENE 6 reagent (Roche Molecular Chemicals; Indianapolis IN) with plasmids encoding 1) the retroviral library, 2) MoMuLV gag and pol genes (pCMV-GP<sub>(Sal)</sub>) and 3) the VSV-G glycoprotein (see Tranque et al.,  
5 *supra*, 1998; Naldini et al., *supra*, 1996). After 48 hr, retroviral particles were recovered from culture supernatant, filtered through a 0.45 µm membrane, and then used to infect B104 rat neural tumor cells (Bottenstein and Sato, Proc. Natl. Acad. Sci., USA 76:514-517, 1979).

10 Approximately 2 x 10<sup>6</sup> COS1 cells were transfected, and approximately the same number of B104 cells were subsequently infected. After 72 hr, cells were harvested and sorted by FACS on a FACSVantage SE (Becton Dickinson; San Jose CA). EGFP was excited with an argon laser tuned to 488 nm and fluorescence was recorded through a 530 nm bandpass filter. ECFP was excited with a krypton/argon  
15 laser tuned to 457 nm, and fluorescence was measured through a 495 nm bandpass filter. As controls for the FACS, B104 cells were infected with the following reference viruses: the parent vector (MESV/EGFP/ECFP/RSVPro; SEQ ID NO: 16), a virus encoding EGFP, a virus encoding ECFP, and a virus that contains the IRES from the encephalomyocarditis virus (EMCV) in the intercistronic region of the  
20 parent vector.

Cells co-expressing both EGFP and ECFP were isolated and returned to culture for 14 days. These cells were then resorted, and high co-expressors were isolated and further expanded in culture for 5 to 7 days. Genomic DNA was prepared  
25 using a QiaAmp DNA miniprep kit (Qiagen). Intercistronic sequences were amplified by PCR using flanking primers, and cloned into the intercistronic region of RPh, which is a dicistronic vector that encodes *Renilla* luciferase protein as the first cistron and *Photinus* luciferase protein as the second cistron (Example 1; Chappell et al., *supra*, 2000). B104 cells were transiently co-transfected with the dual luciferase  
30 vector and with a vector expressing β-galactosidase, and luciferase and β-galactosidase assays were performed (see Example 1). *Photinus* luciferase activity values were normalized for transfection efficiency by means of β-galactosidase

activity, and were then normalized to the activity of the RPh parent vector (first library) or of RPh containing the  $(S_{III}/S_{II})_5\beta$  oligonucleotide as a spacer control (second library).

- 5            Sequences of the oligonucleotide inserts were determined using an ABI system sequencer (PE Biosystems, Foster City, CA), and were compared using the Clustal X multiple sequence alignment program (Thompson et al., Nucl. Acids Res. 25:4876-4882, 1997), and with the BestFit program from the Genetics Computer Group software package (Devereux et al., Nucl. Acids Res. 12:387-395, 1984).
- 10          Sequence matches were evaluated by comparing BestFit quality scores to those obtained when the selected sequences were randomly shuffled 10 times and compared to 18S rRNA. Secondary structure predictions were made using mfold version 3.0 (Zuker et al., in "RNA Biochemistry and Biotechnology" (ed. Clark; Kluwer academic publishers 1999), pages 11-43; Mathews et al., J. Mol. Biol. 288:911-940,
- 15          1999). Northern blot analysis was performed as described in Example 1 using a riboprobe encompassing the entire coding region of the *Photinus* luciferase gene.

- The retroviral library containing the random 18 nucleotide inserts was examined. This library, derived from  $2.5 \times 10^5$  retroviral plasmids was used to infect
- 20          approximately  $2 \times 10^6$  rat B104 neural tumor cells. After 72 hr, cells that co-expressed both EGFP and ECFP, corresponding to approximately 0.5% of the cells, were isolated by FACS. These cells were cultured for 14 days, sorted again by FACS, and high co-expressors, corresponding to approximately 4% of cells, were collected and grown. The twice sorted cells were compared to cells that had been
- 25          infected with the virus that contained the EMCV IRES between the EGFP and ECFP genes. Both cell populations showed variable expression suggesting that IRES activity can vary among individual cells, perhaps reflecting cell cycle differences in the population.

- 30          Intercistronic sequences contained within the population of twice sorted cells were isolated by genomic PCR, and cloned into the intercistronic polylinker of the

RPh vector (see Example 1). This dual luciferase vector has a stable hairpin-forming sequence in the transcribed leader region upstream of the *Renilla* open reading frame. The hairpin structure blocks scanning ribosomes and therefore suppresses translation of the first cistron. Fifty clones were picked at random and plasmid DNA was  
5 prepared, sequenced, and transiently transfected into B104 cells. Of the 45 clones that were successfully sequenced, 39 contained unique 18 nucleotide inserts. The sequences of the other 6 clones were each represented more than once, which may reflect the relatively low complexity of selected sequences in these twice sorted cells.

10 The sequenced clones were tested in transfected cells and most activities were weak or at a background level. However, one sequence, designated intercistronic sequence 1-23 (ICS1-23; SEQ ID NO: 49) demonstrated enhanced *Photinus* luciferase activity approximately 8-fold greater than the control constructs. This level of activity was similar to that observed for one copy of the Gtx IRES module  
15 (Example 1).

A sequence comparison between ICS1-23 (SEQ ID NO: 49) and 18S rRNA (SEQ ID NO: 51) revealed a complementary match between the 3' end of the IRES and 18S rRNA at nucleotides 1311-1324 (Figure 5). This match has a BestFit quality  
20 score that is significantly greater than that obtained with 10 randomized variations of this sequence. To address whether the region of complementarity within ICS1-23 was associated with the IRES activity, the 30 nucleotide ICS1-23 sequence, which includes the 18 nucleotide random sequence together with 12 nucleotides of flanking sequence, was divided into two segments of 15 nucleotides each, ICS1-23a, which  
25 lacked any complementarity to 18S rRNA (see Figure 5, and ICS1-23b (SEQ ID NO: 50) contained the complementary match to 18S rRNA (SEQ ID NO: 51).

As disclosed in Example 1, multiple linked copies of the Gtx IRES module (SEQ ID NO: 2) were more active than the corresponding monomer. Accordingly,  
30 multimers of each segment of ICS1-23 were synthesized, with each repeated segment separated by nine adenosine nucleotides (poly(A)<sub>9</sub>). Three linked copies of the

ICS1-23a segment (see Figure 5) did not enhance *Photinus* luciferase expression. In contrast, constructs containing three and five linked copies of ICS1-23b (SEQ ID NO: 51) enhanced *Photinus* luciferase activity as compared to ICS1-23. These results indicate that the sequence of ICS1-23 that shares complementarity with 18S rRNA has IRES activity. Northern blot analysis of RNA from cells expressing the five linked copies of ICS1-23b revealed a single hybridizing band corresponding in size to the full length dicistronic mRNA, thus confirming that ICS-23b did not enhance *Photinus* luciferase activity by other mechanisms such as alternative splicing or by functioning as a promoter.

The second retroviral library, which contained random 9 nucleotide segments separated by a poly(A)<sub>9</sub> spacer in the intercistronic region of the encoded dicistronic mRNA, was examined in order to identify smaller translational regulatory elements. Incorporation of the spacer sequence was based on the determination that the Gtx IRES module (SEQ ID NO: 2), when present in multiple copies separated by the poly(A)<sub>9</sub> spacer, exhibited greater IRES activity than a single copy of the module (see Example 1).

Approximately  $2 \times 10^6$  B104 cells were transduced with the second retroviral library, which was derived from  $1.5 \times 10^5$  retroviral plasmids. Approximately 0.3% of the cells were selected by FACS, and cultured and sorted a second time. Approximately 3% of the latter cells were high co-expressors. The oligonucleotide inserts were recovered by genomic PCR and shotgun cloned into the intercistronic region of the RPh. One hundred clones were picked at random and 84 were successfully sequenced, yielding 37 different sequences. Fifteen of the sequences were represented two or more times, indicating that the complexity of the sequences represented in these twice sorted cells was somewhat lower than that of the first library. When tested by transient transfection in B104 cells, most sequences enhanced *Photinus* luciferase activity weakly (about 2-fold or less above background), and none were as active as ICS1-23 (SEQ ID NO: 49).

Six of the sequences, which were isolated four or more times from the twice sorted cells, were examined further. Each of these sequences contained two 9 nucleotide segments, which were tested individually as five linked copies. One of these constructs, containing a 9 nucleotide segment designated ICS2-17.2 (TCCGGTCGT; SEQ ID NO: 52), showed enhanced *Photinus* luciferase activity. In contrast to the five linked copies of ICS2-17.1, the other 9 nucleotide segment contained within selected sequence ICS2-17 did not have IRES activity. RNA analysis confirmed that a single transcript was produced from the construct, and that the increase in *Photinus* luciferase activity was derived from an intact dicistronic mRNA. These results indicate that ICS2-17.2 (SEQ ID NO: 52) functions as an IRES.

Five linked copies of both ICS1-23b and ICS2-17.2 also were examined using the 5' UTR of a monocistronic reporter mRNA. In 7 cell lines tested, (ICS1-23b)<sub>5</sub> blocked translation by approximately 70% and (ICS2-17.2)<sub>5</sub> slightly enhanced translation. In both cases, mRNA levels appeared to be unaffected. This result indicates that ICS1-23b and ICS2-17.2 function as IRES elements in the dicistronic mRNAs, but not as transcriptional promoters or enhancers. As with ICS1-23b, sequence comparisons identified a complementary match between ICS2-17.2 and 18S rRNA with a BestFit quality score that is significantly greater than that obtained with 10 randomized variations of the this sequence.

The activity of the selected ICS1-23b (SEQ ID NO: 51) and ICS2-17.2 (SEQ ID NO: 52) IRES modules was examined in additional cell lines to determine whether they were active in cell types other than the B104 neuroblastoma cells. A construct of five linked copies of each module was active in each of the cells line tested, including rat glioma C6 cells, human neuroblastoma SK cells, mouse neuroblastoma N2a cells, mouse NIH-3T3 fibroblasts, human cervical carcinoma HeLa cells, normal rat kidney NRK cells, and mouse muscle myoblast C2C12 cells. The activities of these synthetic IRESes varied as much as ten-fold between cell lines, and also varied with respect to each other. However, the pattern of activity of the ICS-23b (SEQ ID

NO: 51) module in the different cell lines tested was similar to that observed for ten linked copies of the Gtx IRES module (SEQ ID NO: 2).

These results demonstrate that relatively small discrete nucleotide sequences  
5 can act as translational regulatory elements, including as IRES elements, which  
mediate cap-independent translation. Furthermore, the two IRES modules identified  
in this Example were selected from only a minute sampling of the total complexity of  
the random oligonucleotides. Thus, it is likely that screening a more complex library  
of random oligonucleotide will identify additional short nucleotide sequences having  
10 IRES or other translational regulatory activity.

It is remarkable that each of the short IRES element disclosed herein,  
including the Gtx IRES (SEQ ID NO: 2), the ICS1-23b IRES (SEQ ID NO: 51), and  
the ICS2-17.2 IRES (SEQ ID NO: 52) can promote internal initiation. Each of these  
15 three IRES modules contain a complementary match to different segments of 18S  
rRNA, suggesting that a direct interaction occurs between the IRES module and the  
40S ribosomal subunit via base pairing to 18S rRNA. Alternatively, one or more of  
the IRES modules may recruit 40S ribosomal subunits by interacting with a protein  
component of the translational machinery, for example, a ribosomal protein, an  
20 initiation factor, or some other bridging protein. The ability to initiate translation  
internally by binding to an initiation factor has been reported, wherein an iron  
response element (IRE) and the bacteriophage  $\lambda$  transcriptional anti-terminator box B  
element were both demonstrated to function as IRESes in the presence of fusion  
proteins between the appropriate binding protein for these RNA elements and eIF4G  
25 (DeGregorio et al., *EMBO J.* 18:4865-4874, 1999). However, the lack of appreciable  
sequence similarities between the IRES modules disclosed herein and cellular IRESes  
in general suggests that a wide variety of nucleotide sequences can function in  
internal translation initiation, and suggests that different sequences recruit  
pre-initiation complexes by different mechanisms.



The observation that synthetic IRESes comprising multimers of ICS1-23b, ICS2-17.2, or the Gtx IRES module show enhanced IRES activity as compared to the corresponding monomers suggest that multiple copies of the IRES module may increase the probability of recruiting 40S ribosomal subunits. A similar observation  
5 has been made for eIF4G tethered to the IRE-binding protein, where there was an approximately linear increase in translation when the number of IRE binding sites was increased from one site to three (DeGregorio et al., *supra*, 1999).

An arresting feature of cellular IRESes, including the disclosed IRES modules,  
10 is their variable potency in different cell types. As such, selection for IRESes in a variety of cell types can provide a means to identify additional elements having cell-specific and tissue-specific activities. If ribosomal recruitment requires direct interaction of IRESes with 18S rRNA, variations in efficiency may reflect differences in the accessibility of particular segments of 18S rRNA in different cell types.  
15 Alternatively, some IRES modules may require or may be blocked by binding proteins that are differentially expressed in various cell types. Such possibilities can be distinguished by determining which proteins or components of the translation machinery bind to particular IRES sequences in various differentiated cells. In view of the modular nature of cellular IRES, combinations of synthetic IRESes can be  
20 constructed and elements having desirable regulatory actions can be selected. Such a combinatorial approach can be used to construct synthetic IRESes having variable translational regulatory activity, for example; highly restricted or widespread translational activity.

#### 25 **D. Additional Synthetic IRES Elements**

A library of synthetic oligonucleotides containing two random 9 nucleotide (Ran9) sequences also was examined for IRES activity. The Ran9 oligonucleotides were in a construct that contained, from the 5' to the 3' end, 62 nucleotides of the  $\beta$ -globin leader, a poly(A)<sub>9</sub> sequence, the first Ran9 oligonucleotide, a poly(A)<sub>9</sub> spacer,  
30 the second Ran9 oligonucleotide, a poly(A)<sub>9</sub> sequence, and 44 nucleotides of the  $\beta$ -globin leader (see, for example, SEQ ID NO: 53), which includes specific nucleotides corresponding to the two Ran9 positions; see below).

Thirty-six of the constructs that exhibited IRES activity are shown as SEQ ID NOS: 53 to 88. In addition, the individual IRES modules from these constructs are shown as SEQ ID NOS: 89 to 160.

5

#### EXAMPLE 5

##### **IDENTIFICATION OF IRES ACTIVITY IN 5' UTRs OF MAMMALIAN RNAs**

IRES elements also were identified in the 5' transcribed leader sequences of the mRNAs that encode the rat amyloid precursor protein (APP; Rogers et al., J. Biol. Chem. 274:6421-6431, 1999, which is incorporated herein by reference) and the beta-site APP cleaving enzyme (Bace; Yan et al., Nature 402:533-7, 1999, which is incorporated herein by reference). In addition, IRES activity was associated with the 5' UTR of the human FMR mRNA. These IRES elements were identified and characterized in the intercistronic region of a dual luciferase dicistronic mRNA, as disclosed above.

15

The 5' transcribed leader sequence of the mRNA encoding rat APP is shown as SEQ ID NO: 161. The 5' transcribed leader sequence of the mRNA encoding rat Bace is shown as SEQ ID NO: 162. The 5' untranslated region of the human FMR mRNA is shown as SEQ ID NO: 163. Using the methods as disclosed in Examples 1 and 6, translational regulatory elements of these 5' UTRs, including IRES modules, readily can be identified and isolated.

20

#### EXAMPLE 6

##### **IDENTIFICATION OF IRES MODULE IN MOUSE Rbm3 5'UTR**

IRES activity also was detected in the 728 nucleotide 5' UTR of the mouse Rbm3 mRNA (SEQ ID NO: 164, which includes ATG initiator methionine). In addition, fragments of the Rbm3 5' UTR had IRES activity, including the following sequences: nucleotides 1 to 97, nucleotides 199 to 299, nucleotides 299 to 406, nucleotides 400 to 501, nucleotides 419 to 501, nucleotides 440 to 460, nucleotides 500

30

to 601, nucleotides 600 to 728, nucleotides 630 to 650, nucleotides 650 to 670 and nucleotides 690 to 710 of SEQ ID NO: 164.

To further characterize the Rbm3 5' UTR, deletions were made from the 5' and 3' ends and examined using the dicistronic RPh vector (see Example 1). Figure 6 shows the sequences of the various deletion constructs (SEQ ID NOS: 165 to 190; note that adenosine residues were added to 3' end of 3' deletion mutants to maintain relative distance to naturally occurring Rbm3 initiator AUG codon) and the IRES activity associated with each (shown as the R:P ratio, normalized to 1.0 for the activity of the parent RPh vector). The deletion studies indicated that IRES activity a core element comprising the sequence TTTATAATTTCTTCTTCCAGAA (SEQ ID NO: 191) conferred IRES activity. In addition, the sequence GAAAGCTTG (SEQ ID NO: 192), which is positioned beginning 8 nucleotides downstream of the core IRES element enhances the activity of the IRES module by about 10-fold, but does not have IRES activity on its own. These results demonstrate that a core IRES module is present in the Rbm3 5' UTR and, together with the results disclosed above, indicate that small IRES modules can be present in a variety of eukaryotic mRNA molecules.

The deletion studies of the Rbm3 5' UTR also indicated that nucleotide sequences in the 5' UTR may inhibit IRES activity. Analysis of the deletion mutants revealed an Rbm3 IRES inhibitory sequence, GGTACATTTTTTTTC (SEQ ID NO: 193), which is positioned about 21 nucleotides downstream of the IRES module and inhibited internal initiation of translation facilitated by the nearby IRES module. In addition, a second inhibitory sequence, ATGCGGAATGATTTATAA (SEQ ID NO: 194), which partially overlaps the 5' end of the Rbm3 IRES module, contains a short open reading frame that inhibits internal initiation only when the Rbm3 initiation codon is mutated. This result indicates that a translational inhibitory element can be masked by an open reading frame.

**EXAMPLE 7****IRES MODULES BASED ON RIBOSOMAL RNA STRUCTURE**

This example demonstrates that synthetic oligonucleotides having IRES activity can be designed based on the structure of ribosomal RNA molecules.

5

It is not present clear how cellular IRESes function. As disclosed herein, however, cellular IRESes exist as modular structures composed of short, independent oligonucleotides, including oligonucleotide that are complementary to 18S rRNA. These results indicate that recruitment of ribosomal subunits by IRES modules is directed by base pairing of the IRES element to the rRNA within the ribosomal subunit.

The 9 nucleotide Gtx IRES module (SEQ ID NO: 2) is 100% complementary to an oligonucleotide sequence of 18S rRNA, and was tested as an IRES module based on this observation. In addition, the ability of the Gtx IRES module (SEQ ID NO: 2) to recruit 40S ribosomal subunits by base pairing to 18S rRNA was examined. Nitrocellulose filter-binding and electrophoretic mobility gel shift assays established a physical link between the 9 nucleotide Gtx IRES module (SEQ ID NO: 2) and dissociated ribosomal subunits, but not with other components of cell lysates. Transfection studies using dicistronic constructs that contained the Gtx IRES module (SEQ ID NO: 2) or mutations of this sequence (see Figure 2) demonstrated that internal initiation was maximal with a mutant module sharing 7 nucleotides of complementarity with 18S rRNA, and that as the degree of complementarity was progressively increased or decreased, IRES activity was decreased and, ultimately, lost. When tested in the 5' or 3' UTR of a monocistronic mRNA, sequences that enhanced internal initiation also functioned as translational enhancers. However, only those sequences with increased complementarity to 18S rRNA inhibited both internal initiation and translation in monocistronic mRNAs. This inhibition appeared to involve stable interactions between the mRNA and 40S ribosomal subunits as determined by polysome analysis. These results indicate that internal initiation of translation can occur at short nucleotide sequences by base pairing to 18S rRNA.

30

Sequence analysis of the IRES-modules recovered from the selection studies showed that most of the selected sequences contained complementary sequence matches of 8 to 9 nucleotides to different regions of the 18S rRNA (Figure 7).

5 Furthermore, many of the matches are to un-base paired regions of the rRNA (see Figure 7B). Moreover, in some cases, several selected sequences with slightly different sequences, were complementary to the same region of the 18S rRNA (see, also, Owens et al., 2001, which is incorporated herein by reference). These results indicate that synthetic translational regulatory elements can be designed based on  
10 rRNA sequences such as those set forth in SEQ ID NOS: 195-197, particularly to un-base paired rRNA sequences, which can be predicted using methods as disclosed herein, such that the synthetic translational regulatory elements are complementary to a selected rRNA target sequence.

15 Although the invention has been described with reference to the above examples, it will be understood that modifications and variations are encompassed within the spirit and scope of the invention. Accordingly, the invention is limited only by the following claims.

What is claimed is:

1. A synthetic translational regulatory element, comprising at least one  
oligonucleotide consisting of about 6 to 125 ribonucleotides, or a deoxyribonucleotide  
5 sequence encoding said oligonucleotide,  
wherein said oligonucleotide has a translational regulatory activity selected from  
translational enhancing activity, translational inhibitory activity, internal ribosome entry  
site (IRES) activity and a combination thereof, and  
wherein the synthetic translational regulatory element has translational regulatory  
10 activity in a eukaryotic cell.
2. The synthetic translational regulatory element of claim 1, wherein said  
oligonucleotide consists of about 8 to 100 ribonucleotides.
- 15 3. The synthetic translational regulatory element of claim 1, wherein said  
oligonucleotide consists of about 9 to 50 ribonucleotides.
4. The synthetic translational regulatory element of claim 1, wherein said  
oligonucleotide is encoded by a nucleotide sequence selected from any of SEQ ID  
20 NOS: 42 to 46, 49, 50, 52, and 89 to 160.
5. The synthetic translational regulatory element of claim 1, comprising at least  
two of said oligonucleotides,  
wherein the at least two oligonucleotides are operatively linked to each other,  
25 and  
wherein each of the at least two oligonucleotides independently is the same or  
different from each other.
6. The synthetic translational regulatory element of claim 5, wherein each of the  
30 at least two oligonucleotides is encoded by a nucleotide sequence selected from any of  
SEQ ID NOS: 42 to 46, 49, 50, 52, and 89 to 160.

7. The synthetic translational regulatory element of claim 5, comprising five of said oligonucleotides, which are operatively linked to each other.

8. The synthetic translational regulatory element of claim 5, comprising ten of said oligonucleotides, which are operatively linked to each other.

9. The synthetic translational regulatory element of claim 5, comprising about 2 to 75 of said oligonucleotides, which are operatively linked to each other.

10. The synthetic translational regulatory element of claim 5, comprising about 10 to 50 of said oligonucleotides, which are operatively linked to each other.

11. The synthetic translational regulatory element of claim 5, wherein each of said at least two oligonucleotides is separated from each other by a spacer nucleotide sequence, wherein said spacer nucleotide sequence consists of about 1 to 100 ribonucleotides.

12. The synthetic translational regulatory element of claim 5, which is encoded by a nucleotide sequence selected from any of SEQ ID NOS: 53 to 88.

13. The synthetic translational regulatory element of claim 1, which has IRES activity.

14. The synthetic translational regulatory element of claim 1, which has translational enhancing activity.

15. The synthetic translational regulatory element of claim 1, wherein said oligonucleotide is complementary to an oligonucleotide sequence of a ribosomal RNA.

16. The synthetic translational regulatory element of claim 15, wherein said oligonucleotide is derived from an oligonucleotide encoded by any of SEQ ID NOS: 2, 30, 32, 34, 36, 38, 40 and 50.

17. A vector, comprising the synthetic translational regulatory element of claim 1.

5           18. The vector of claim 17, which is an expression vector.

19. The expression vector of claim 18, which comprises a translation initiation site, wherein the translational regulatory element has translational enhancing activity.

10           20. The expression vector of claim 19, further comprising a translation start codon operatively linked to the translation initiation site.

21. The expression vector of claim 19, further comprising an expressible polynucleotide,  
15           wherein the translational regulatory element is operatively linked to the expressible polynucleotide, and  
              wherein the translational regulatory element has translational enhancing activity or IRES activity.

20           22. The expression vector of claim 21, wherein the expressible polynucleotide comprises at least one cistron.

23. The expression vector of claim 22, wherein the expressible polynucleotide is polycistronic.

25           24. A host cell containing the synthetic translational regulatory element of claim 1.



25. An isolated translational regulatory element, comprising at least one 5' untranslated region (5' UTR) of a eukaryotic messenger RNA (mRNA) or an oligonucleotide portion thereof, or a deoxyribonucleotide sequence encoding said 5' UTR or oligonucleotide portion thereof,

- 5            wherein said 5' UTR or oligonucleotide portion thereof has an activity selected from translational enhancing activity, internal ribosome entry site (IRES) activity, translational inhibitory activity, and a combination thereof, and
- wherein said translational regulatory element has translational regulatory activity in a eukaryotic cell.

10

26. The isolated translational regulatory element of claim 25, wherein said 5' UTR is encoded by a nucleotide sequence selected from any of SEQ ID NOS: 1, 23 to 29, 161, 162, and 164, and

- wherein said 5' UTR or oligonucleotide portion thereof has an activity selected
- 15    from translational enhancing activity and IRES activity.

27. The isolated translational regulatory element of claim 26, wherein said oligonucleotide portion is encoded by a nucleotide sequence selected from any of nucleotides 1 to 40, 1 to 81, 1 to 120, 41 to 81, 14 to 196, 80 to 120, 80 to 196, 120, to

20    166, and 120 to 196 of SEQ ID NO: 1.

28. The isolated translational regulatory element of claim 26, wherein said oligonucleotide portion is encoded by SEQ ID NO: 2.

- 25            29. The isolated translational regulatory element of claim 28, wherein said oligonucleotide portion encoded by SEQ ID NO: 2 is linked at its 5' end or 3' end or both 5' and 3' ends to a spacer nucleotide sequence, wherein said spacer nucleotides sequence consists of about 1 to 100 ribonucleotides.

- 30            30. The isolated translational regulatory element of claim 29, which is encoded by a nucleotide sequence selected from SEQ ID NOS: 4, 5, 12, and 20 to 22.

31. The isolated translational regulatory element of claim 25, comprising at least two 5' UTRs or oligonucleotide portions thereof,

wherein the at least two 5' UTRs or oligonucleotide portions thereof are operatively linked to each other, and

5 wherein each of the at least two 5' UTRs or oligonucleotide portions thereof independently is the same or different from each other.

32. The isolated translational regulatory element of claim 31, comprising at least two oligonucleotide portions encoded by SEQ ID NO: 2, which are operatively linked to  
10 each other.

33. The isolated translational regulatory element of claim 31, comprising five oligonucleotide portions encoded by SEQ ID NO: 2, which are operatively linked to  
15 each other.

34. The isolated translational regulatory element of claim 31, comprising ten oligonucleotide portions encoded by SEQ ID NO: 2, which are operatively linked to each other.

20 35. The synthetic translational regulatory element of claim 31, comprising about 2 to 75 of said 5' UTRs or oligonucleotide portions thereof, which are operatively linked to each other.

36. The synthetic translational regulatory element of claim 31, comprising about  
25 10 to 50 of said 5' UTRs or oligonucleotide portions thereof, which are operatively linked to each other.

37. The isolated translational regulatory element of claim 31, wherein each of the at least two 5' UTRs or oligonucleotide portions thereof is separated from each other  
30 by a spacer nucleotide sequence, wherein said spacer nucleotide sequence consists of about 1 to 100 ribonucleotides.

38. The isolated translational regulatory element of claim 37, consisting of two oligonucleotide portions encoded by SEQ ID NO: 2.
39. The isolated translational regulatory element of claim 38, which is encoded  
5 by a nucleotide sequence selected from any of SEQ ID NOS: 6 to 11 and 13 to 15.
40. The isolated translational regulatory element of claim 26, wherein said oligonucleotide portion is encoded by a nucleotide sequence selected from any of nucleotides 1 to 250, 100 to 508, 160 to 508, 250 to 508, 375 to 508, 429 to 508, 481  
10 to 508, and 250 to 390 of SEQ ID NO: 29.
41. The isolated translational regulatory element of claim 26, wherein said oligonucleotide portion is encoded by a nucleotide sequence selected from any of  
15 SEQ ID NOS: 165 to 169, 171 to 176, and 182 to 190.
42. The isolated translational regulatory element of claim 26, wherein said oligonucleotide portion is encoded by a nucleotide sequence selected from SEQ ID NOS: 191 and 192.
- 20 43. The isolated translational regulatory element of claim 25,  
wherein said 5' UTR is encoded by a nucleotide sequence selected from any of  
SEQ ID NOS: 1, 23 to 29, 161, 162, and 164, and  
wherein said oligonucleotide portion thereof has translational inhibitory activity.
- 25 44. The isolated translational regulatory element of claim 43, wherein said oligonucleotide portion is encoded by a nucleotide sequence selected from nucleotides 120 to 196 and 167 to 196 of SEQ ID NO: 1.
- 30 45. The isolated translational regulatory element of claim 43, wherein said oligonucleotide portion is encoded by a nucleotide sequence selected from nucleotides 1 to 100 and 100 to 160 of SEQ ID NO: 29.

46. The isolated translational regulatory element of claim 43, wherein said oligonucleotide portion is encoded by a nucleotide sequence selected from SEQ ID NO: 193 and SEQ ID NO: 194.

5           47. The isolated translational regulatory element of claim 25, wherein said 5' UTR or oligonucleotide portion thereof is complementary to an oligonucleotide sequence of a ribosomal RNA.

          48. The isolated translational regulatory element of claim 47, wherein said  
10 oligonucleotide portion is encoded by any of SEQ ID NOS: 2, 30, 32, 34, 36, 38, 40 and 50.

          49. A vector, comprising the translational regulatory element of claim 25.

15           50. The vector of claim 49, which is an expression vector.

          51. The expression vector of claim 49, further comprising an expressible polynucleotide,

          wherein the translational regulatory element is operatively linked to the  
20 expressible polynucleotide, and

          wherein the translational regulatory element has transcriptional enhancing activity or IRES activity.

          52. A host cell containing the translational regulatory element of claim 25.  
25

          53. A kit, comprising the synthetic translational regulatory element of claim 1.

          54. The kit of claim 53, comprising a plurality of synthetic translational regulatory elements, which are the same as or different from each other.  
30

55. The kit of claim 54, wherein each of the synthetic translational regulatory elements in the plurality comprises a flanking sequence independently at a 5' end or a 3' end or both 5' and 3' ends, and

wherein the flanking sequences provide a means to operatively link two or more  
5 synthetic translational regulatory elements in the plurality to each other.

56. A recombinant nucleic acid molecule, comprising the synthetic translational regulatory element of claim 1 operatively linked to an expressible polynucleotide.

10 57. The recombinant nucleic acid molecule of claim 56, wherein the expressible polynucleotide comprises a cistron, and wherein the synthetic translational regulatory element has translational enhancing activity or IRES activity.

58. The recombinant nucleic acid molecule of claim 56, wherein the expressible  
15 polynucleotide comprises, in operative linkage in a 5' to 3' orientation, a first cistron, a spacer nucleotide sequence, and a second cistron,

wherein the synthetic translational regulatory element is has IRES activity, and

wherein the synthetic translational regulatory element is operatively linked to the second cistron.

20

59. The recombinant nucleic acid molecule of claim 56, wherein the synthetic translational regulatory element comprises at least two operatively linked oligonucleotides having translational regulatory activity.

25 60. The recombinant nucleic acid molecule of claim 56, wherein the expressible polynucleotide encodes at least one polypeptide.

61. The recombinant nucleic acid molecule of claim 60, wherein the at least one polypeptide is an enzyme.

30

62. The recombinant nucleic acid molecule of claim 61, wherein the enzyme is selected from  $\beta$ -galactosidase,  $\beta$ -glucuronidase, luciferase, alkaline phosphatase, glutathione S-transferase, chloramphenicol acetyltransferase, guanine xanthine phosphoribosyltransferase, and neomycin phosphotransferase.

5

63. The recombinant nucleic acid molecule of claim 60, wherein the at least one polypeptide is selected from a viral polypeptide and a bacterial polypeptide.

64. The recombinant nucleic acid molecule of claim 60, wherein at least one  
10 the polypeptide comprises an epitope expressed by a pathogenic organism.

65. The recombinant nucleic acid molecule of claim 60, wherein the at least one polypeptide is selected from a growth factor, a hormone and a receptor for a growth factor or a hormone.

15

66. The recombinant nucleic acid molecule of claim 60, wherein the expressible polynucleotide encodes two polypeptides.

67. A method of producing a genetically modified cell that exhibits altered  
20 expression of a polypeptide, the method comprising introducing a synthetic translational regulatory element of claim 1 into a cell, whereby the synthetic translational regulatory element operatively linked to a nucleotide sequence encoding a polypeptide, thereby producing a genetically modified cell that exhibits altered expression of a polypeptide.

25

68. The method of claim 67, wherein the synthetic translational regulatory element is operatively linked to the nucleotide sequence prior to introducing the synthetic translational regulatory element into the cell, wherein the synthetic translational regulatory element has translational enhancing activity or IRES activity.

30

69. The method of claim 67, wherein the polypeptide is a reporter polypeptide.

70. The method of claim 67, wherein the polypeptide is a toxin.

71. The method of claim 67, wherein the polypeptide is a therapeutic agent.

5

72. The method of claim 66, wherein the synthetic translational regulatory element is stably maintained in the cell.

73. The method of claim 66, wherein the synthetic translational regulatory  
10 element is integrated in the cell genome.

74. The method of claim 73, wherein the nucleotide sequence is a sequence of an endogenous gene in the cell genome.

15 75. The method of claim 73, wherein the synthetic translational regulatory element has translational inhibitory activity.

76. The method of claim 73, wherein the synthetic translational regulatory element has translational enhancing activity or IRES activity

20

77. A genetically modified eukaryotic cell produced by the method of claim 73.

78. A transgenic non-human eukaryotic organism, comprising the genetically  
25 modified cell of claim 77.

79. A cell or tissue obtained from the transgenic non-human eukaryotic organism of claim 78.

30 80. A cDNA or genomic DNA library prepared from the transgenic non-human eukaryotic organism of claim 78, or from a cell or tissue obtained from said transgenic non-human eukaryotic organism.

81. A method of altering translational activity in a eukaryotic cell, the method comprising introducing into the cell a synthetic translational regulatory element of claim 1, whereby the synthetic translational regulatory element interacts with a translation regulatory factor in the cell, thereby altering translational activity in the eukaryotic cell.

82. A method of altering translational activity in a eukaryotic cell, the method comprising introducing into the cell an isolated translational regulatory element of claim 25, whereby the isolated translational regulatory element interacts with a translation regulatory factor in the cell, thereby altering translational activity in the eukaryotic cell.

83. The method of claim 82, wherein the isolated translational regulatory element comprises an isolated translational regulatory element of claim 25, whereby translational activity in the cell is decreased.

84. The method of claim 83, wherein the isolated translational regulatory element comprises a synthetic translational regulatory element of claim 43, whereby translational activity in the cell is decreased.

85. A method of improving protein yield by a eukaryotic cell, comprising introducing into the cell a recombinant nucleic acid molecule comprising a synthetic translational regulatory element of claim 1 operatively linked to an expressible polynucleotide,

wherein the translational regulatory element has an activity selected from translational enhancing activity and IRES activity, and

wherein the expressible polynucleotide is expressed in the cell.

86. The method of claim 85, wherein the expressible polynucleotide comprises a first cistron encoding a polypeptide that enhances protein stability or cell viability.



87. The method of claim 86, wherein the expressible polynucleotide further comprises a second cistron encoding a polypeptide of interest,

wherein the second cistron is operatively linked to the first cistron, and

5 wherein the expressible polynucleotide comprises an IRES element, which is operatively linked to the first cistron or the second cistron or both.

88. The method of claim 86, wherein the polypeptide that enhances protein stability or cell viability is selected from a chaperone protein, a heat shock protein, a  
10 protein having anti-oxidant activity, a protease inhibitor, and a phosphatase inhibitor.

89. The method of claim 86, wherein the polypeptide that enhances protein stability or cell viability is selected from a caspase inhibitor, a magainin, a defensin, and a cryptdin.

15

90. The method of claim 87, wherein the translational regulatory element comprises a plurality of operatively linked oligonucleotide having translational enhancing activity or IRES activity or both.

20 91. The method of claim 85, wherein the expressible polynucleotide comprises a first cistron encoding a polypeptide that enhances transcription or translation of a polynucleotide in the cell.

92. The method of claim 91, wherein the expressible polynucleotide further  
25 comprises a second cistron encoding a polypeptide of interest,  
wherein the second cistron is operatively linked to the first cistron,  
wherein the expressible polynucleotide comprises an IRES element, which is operatively linked to the first cistron or the second cistron or both, and  
and wherein the a polypeptide that enhances transcription or translation of a  
30 polynucleotide is a polypeptide that enhances transcription or translation of the expressible polynucleotide.

93. The method of claim 91, wherein the polypeptide that enhances transcription or translation of a polynucleotide in the cell is selected from a transcription factor and a translation regulatory factor.

5           94. The method of claim 93, wherein the polypeptide that enhances transcription or translation of a polynucleotide in the cell is a eukaryotic initiation factor.

95. A method of expressing a polypeptide in a eukaryotic cell, comprising:  
10           a) introducing into the cell a recombinant nucleic acid molecule comprising a synthetic translational regulatory element of claim 1 operatively linked to an expressible polynucleotide,  
              wherein the translational regulatory element has an activity selected from translational enhancing activity and IRES activity, and  
15           b) expressing the expressible polynucleotide in the cell.

96. The method of claim 95, wherein the expressible polynucleotide comprises a first cistron encoding a therapeutic polypeptide.

20           97. The method of claim 96, wherein the therapeutic polypeptide is selected from an immunomodulator, a neuromodulator, a hormone, a growth factor, a growth factor receptor, an apoptotic polypeptide, an anti-apoptotic polypeptide, and an antibiotic.

25           98. The method of claim 96, wherein the expressible polynucleotide further comprises a second cistron encoding a polypeptide that facilitates expression or activity of the therapeutic polypeptide,  
              wherein the second cistron is operatively linked to the first cistron, and  
              wherein the expressible polynucleotide comprises an IRES element, which is  
30           operatively linked to the first cistron or the second cistron or both.

99. The method of claim 98, polypeptide that facilitates expression or activity of the therapeutic polypeptide is selected from a transcription factor that increases transcription of the expressible polynucleotide, a translational regulatory factor that increases translation of a polypeptide encoded by the expressible polynucleotide, a  
5 chaperone protein, a protein having anti-oxidant activity, a protease inhibitor, and a phosphatase inhibitor.

100. The method of claim 95, wherein introducing the expressible polynucleotide is cell is performed *ex vivo* or *in vivo*.

10

101. The method of claim 95, wherein the recombinant nucleic acid molecule is contained in a vector.

15

102. The method of claim 101, wherein the vector is a viral vector.

103. The method of claim 102, wherein the viral vector is selected from an adenovirus vector, an adeno-associated virus vector, and a retrovirus vector.

20

104. The method of claim 95, wherein the expressible polynucleotide encodes a reporter polypeptide.

105. The method of claim 104, wherein expression of the reporter polypeptide is detectable in a cell *ex vivo* or *in vivo*.

25

106. The method of claim 104, wherein expression of the reporter polypeptide provide a means to diagnose or monitor the progression of a pathologic condition.

107. A method of identifying a cell, comprising introducing into the cell a recombinant nucleic acid molecule comprising a synthetic translational regulatory element of claim 1 operatively linked to an expressible polynucleotide,

wherein the translational regulatory element has an activity selected from  
5 translational enhancing activity and IRES activity, and

wherein the expressible polynucleotide comprises at least one cistron, which encodes a first reporter polypeptide, and

wherein expression of the reporter polypeptide in the cell provides a means to identify the cell.

10

108. The method of claim 107, wherein the expressible polynucleotide further comprises a second cistron encoding a polypeptide of interest,

wherein the second cistron is operatively linked to the first cistron, and

wherein the expressible polynucleotide comprises an IRES element, which is  
15 operatively linked to the first cistron or the second cistron or both.

109. The method of claim 108, wherein the polypeptide of interest is a second reporter polypeptide.

20 110. The method of claim 109, wherein the second reporter polypeptide is different from the first reporter polypeptide.

111. The method of claim 107, further comprising isolating a cell expressing a reporter polypeptide.

25

112. The method of claim 111, wherein the reporter polypeptide is a cell surface marker, and wherein isolating the cell expressing the cell surface marker comprises using an antibody that specifically binds the cell surface marker.

113. The method of claim 112, wherein the cell surface marker comprises a  
30 peptide tag operatively linked to a cell surface protein.

114. An isolated cell obtained by the method of claim 111.

FIGURE 1

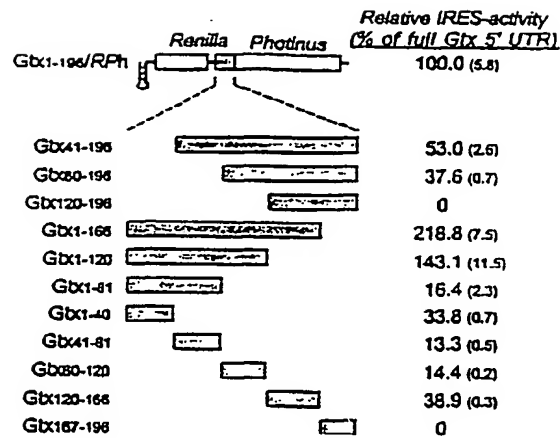


FIGURE 2

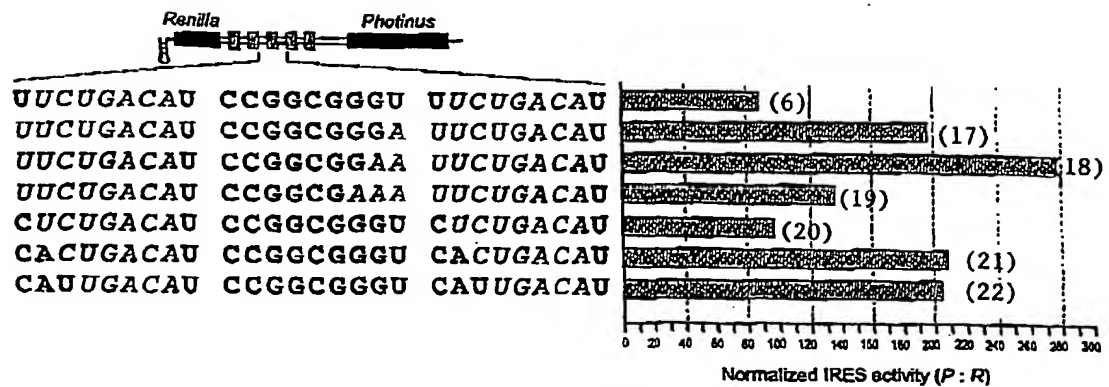


FIGURE 3

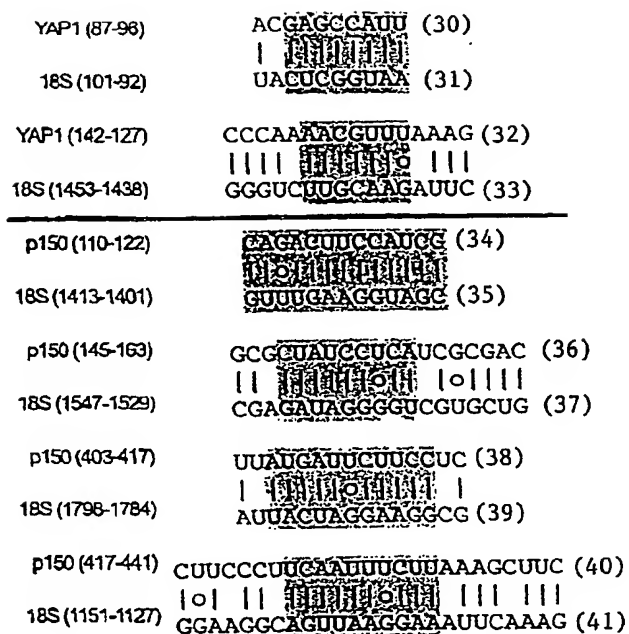


FIGURE 4

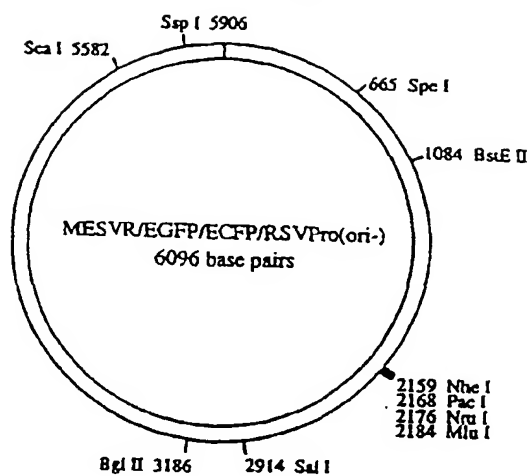
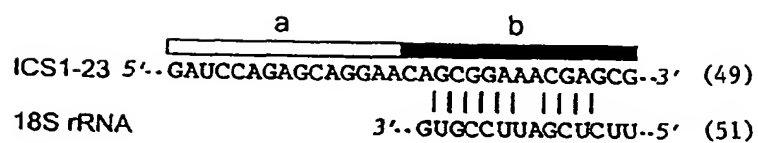
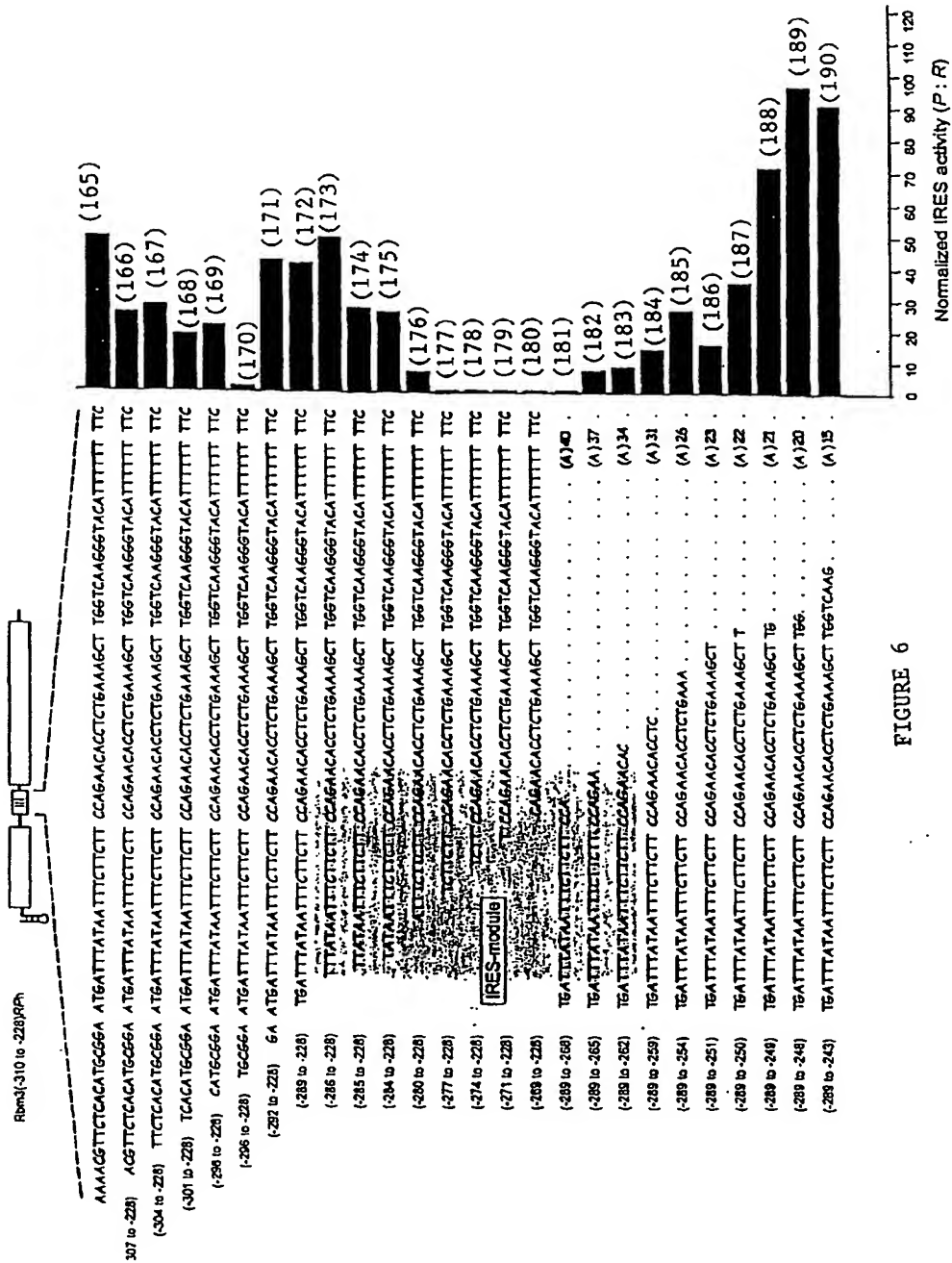


FIGURE 5







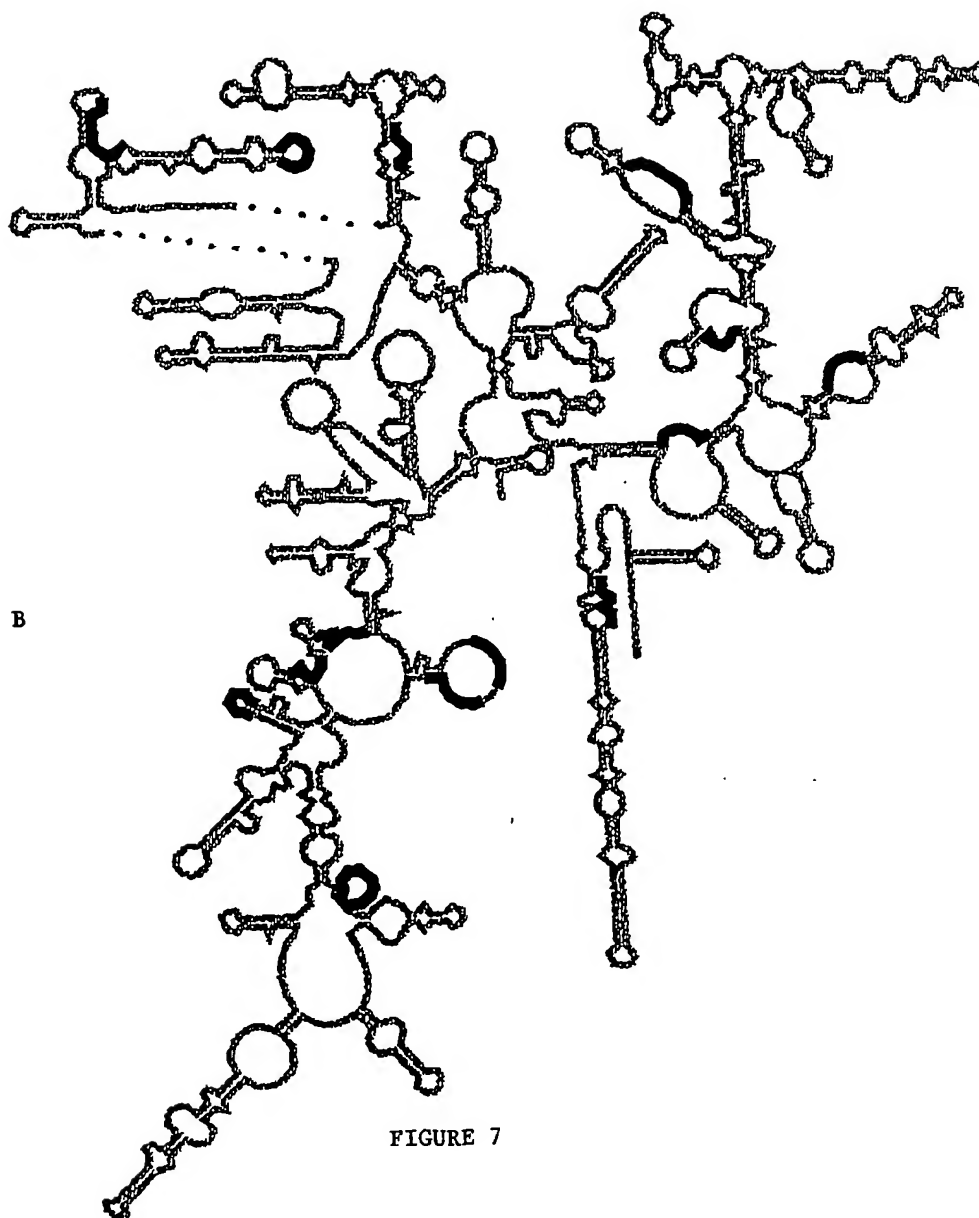
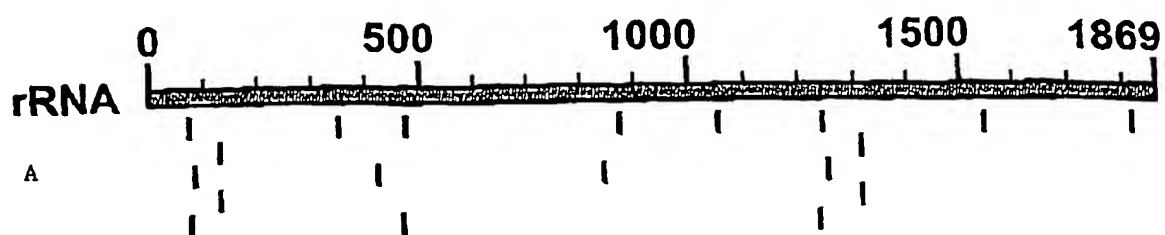


FIGURE 7

1

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THE NEUROSCIENCES INSTITUTE

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<151> 2000-05-30

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<151> 2000-03-02

<150> US 60/178,816

<151> 2000-01-28

<150> US (attorney docket SCRIP1370)

<151> 2001-01-12

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<220>  
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<220>  
 <223> Gtx IRES followed by 25 nt beta-globin

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 <213> ARTIFICIAL

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 spacers followed by 25 nt of the beta globin 5' UT

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 spacers followed by 25 nt of the beta globin 5' UT

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ggtttctgac atccggcggg tgaattcttc tgacatccgg cgggtttctg acatccggcg 120  
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 spacers followed by 25 nt of the beta globin 5' UT

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 spacers followed by 25 nt of the beta globin 5' UT

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 tcacaacccc agaaacagac at 202

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 spacers followed by 25 nt of the beta globin 5' UT

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 tcacaacccc agaaacagac at 202

<210> 12

<211> 106

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<213> ARTIFICIAL

<220>

<223> eight repeats of the 9 nt spacer, upstream of the 9 nt Gtx IRES-element, followed by 25 nt of the b-globin 5' UT

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<223> six repeats of the 9 nt spacer, upstream of two repeats of the 9 nt Gtx IRES-element, spaced by the 9 nt spacer, followed by the 25 nt of the beta-globin 5' UTR

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<211> 106

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 t Gtx IRES-element spaced 54 nt apart with the 9 nt spacer follow  
 ed by the 25 nt beta-globin 5' UT

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tcgtcttcaa	6250

<210> 17  
<211> 117  
<212> DNA  
<213> ARTIFICIAL

<220>  
<223> (SI/Gtx133-141) 5beta/RPh

<400> 17  
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catccggcgg gattctgaca tccggcgggg gactcacaac cccagaaaca gacatcc 117

<210> 18  
<211> 117  
<212> DNA  
<213> ARTIFICIAL

<220>  
<223> (SI/Gtx133-141) 5beta/RPh

<400> 18  
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catccggcgg aattctgaca tccggcgga gactcacaac cccagaaaca gacatcc 117

<210> 19  
<211> 117  
<212> DNA  
<213> *Saccharomyces cerevisiae*

<400> 19  
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catccggcga aattctgaca tccggcgaaa gactcacaac cccagaaaca gacatcc 117

<210> 20  
<211> 117  
<212> DNA  
<213> ARTIFICIAL

<220>  
<223> (SI/Gtx133-141) 5beta/RPh

<400> 20  
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<210> 21  
<211> 117  
<212> DNA  
<213> ARTIFICIAL

<220>

<223> (SI/Gtx133-141) 5beta/RPh

<400> 21

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<210> 22

<211> 117

<212> DNA

<213> ARTIFICIAL

<220>

<223> (SI/Gtx133-141) 5beta/RPh

<400> 22

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catccggcgg gtcattgaca tccggcgggt cattcacaac cccagaaaca gacatcc 117

<210> 23

<211> 151

<212> DNA

<213> Rattus norvegicus

<400> 23

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gcgagtggcc cctagttctg ggagcagagt atcagcatcc cagtcctagt cccgagccta 120

aagcctcgcc tgcccgcca gtgccaggat g 151

<210> 24

<211> 148

<212> DNA

<213> Rattus norvegicus

<400> 24

gttattctcc gaggggcccc acagcccccg ggagctccag gatgaggagt ccggcagctg 60

cctctgggtg cagaagtcca agctgttggg gattgaagtg aagactattt cctgtcatta 120

tagccgtcgc gctgcttctc gacagtcc 148

<210> 25

<211> 216

<212> DNA

<213> Rattus norvegicus

<400> 25

agtgtctctg cgagtagtcc tccctcagcc gcagtctctg ggcctcttca gcttgagcgg 60

cggcgagcct gccacactcg ctaagctcct ccggcaccgc gcaattgcca ctgccactgc 120

cgcttcgcgc ccgctgcagc cgccggctct gaatccttct ggcttcgcgc tcagaggagt 180

tcttagcctg tcccgaaccg taaccccggc gagcag 216

<210> 26  
 <211> 367  
 <212> DNA  
 <213> *Rattus norvegicus*

<400> 26  
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 cgtgaccgcg ggtgcgtcca ctttcggtgc ccagattttt attgatccga accaacctat 180  
 attgtgactg ctggcaagaa ttaagattct tcagcggggc tctaacagag ggagcgctga 240  
 ttgggaagca ttcagtcagg aaattaaaag aaagaagcca gaacatacca ccagcccttt 300  
 gcgtatacca cataacaaac gtcattactt tacaacttga ttaggagaca gtacagagat 360  
 ctgaagg 367

<210> 27  
 <211> 249  
 <212> DNA  
 <213> *Rattus norvegicus*

<400> 27  
 ctccagttct ccccgccac cctacagaaa gtgtctcctg attggctttg aggccgcagg 60  
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 ctggttctgc accgattctg tgetggtccg ggagtgcccg acagcccctg aactaccacc 180  
 cagcattgta caaaccacc cccactctga gccaggctcc accccagcca aggaccctca 240  
 acaccggca 249

<210> 28  
 <211> 178  
 <212> DNA  
 <213> *Saccharomyces cerevisiae*

<400> 28  
 accgattaag cacagtacct ttacgttata tataggattg gtgttttagct ttttttcctg 60  
 agcccctggt tgacttgtgc atgaacacga gccattttta gtttgtttaa gggaagtgtt 120  
 ttgccacca aaacgtttta agaaggaaaa gttgtttctt aaaccatgag tgtgtcta 178

<210> 29  
 <211> 511  
 <212> DNA  
 <213> *Saccharomyces cerevisiae*

<400> 29  
 aatcattttt ttgaaaatta cattaataag gcttttttca atatctctgg aacaacagtt 60  
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 cgtagtggga tgatcatatg atgggcgcta tcctcatcgc gactcgataa cgacgtgaga 180  
 aacgattttt ttttttcttt ttcaccgtat ttttgtgctt cctttttcaa ttatagcttt 240  
 tttttatttt ttttttttct cgtactgttt cactgacaaa agtttttttt caagaaaaat 300  
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 cttgaaattt taccaatctt gatattgtga taatttactt aattatgatt ctctctcttc 420  
 ccttcaattt cttaaagctt cttactttac tccttcttgc tcataaataa gcaaggtaag 480  
 aggacaactg taattaccta ttacaataat g 511

<210> 30  
 <211> 10  
 <212> RNA  
 <213> *Saccharomyces cerevisiae*

<400> 30  
 acgagccauu 10

<210> 31  
 <211> 10  
 <212> RNA  
 <213> *Saccharomyces cerevisiae*

<400> 31  
 aauggcucau 10

<210> 32  
 <211> 16  
 <212> RNA  
 <213> *Saccharomyces cerevisiae*

<400> 32  
 gaaaauugca aaaccc 16

<210> 33  
 <211> 16  
 <212> RNA  
 <213> *Saccharomyces cerevisiae*

<400> 33  
 cuuagaacgu ucuggg 16

<210> 34  
 <211> 13  
 <212> RNA

13

&lt;213&gt; Saccharomyces cerevisiae

&lt;400&gt; 34

cagacuucca ucg

13

&lt;210&gt; 35

&lt;211&gt; 13

&lt;212&gt; RNA

&lt;213&gt; Saccharomyces cerevisiae

&lt;400&gt; 35

cgauggaagu uug

13

&lt;210&gt; 36

&lt;211&gt; 19

&lt;212&gt; RNA

&lt;213&gt; Saccharomyces cerevisiae

&lt;400&gt; 36

gcgcuauccu caucgcgac

19

&lt;210&gt; 37

&lt;211&gt; 19

&lt;212&gt; RNA

&lt;213&gt; Saccharomyces cerevisiae

&lt;400&gt; 37

gucgugcugg ggauagagc

19

&lt;210&gt; 38

&lt;211&gt; 15

&lt;212&gt; RNA

&lt;213&gt; Saccharomyces cerevisiae

&lt;400&gt; 38

uuaugauucu uccuc

15

&lt;210&gt; 39

&lt;211&gt; 15

&lt;212&gt; RNA

&lt;213&gt; ARTIFICIAL

&lt;220&gt;

&lt;223&gt; 18s rRNA

&lt;400&gt; 39

gcggaaggau cauua

15

&lt;210&gt; 40

&lt;211&gt; 25

&lt;212&gt; RNA

&lt;213&gt; Saccharomyces cerevisiae

<400> 40  
cuucccuuca auuucuuaaa gcuuc 25

<210> 41  
<211> 25  
<212> RNA  
<213> Saccharomyces cerevisiae

<400> 41  
gaaacuuaaa ggaauugacg gaagg 25

<210> 42  
<211> 30  
<212> DNA  
<213> ARTIFICIAL

<220>  
<223> IRES-29

<400> 42  
gatccaggca gaggagagga gggacgagcg 30

<210> 43  
<211> 30  
<212> DNA  
<213> ARTIFICIAL

<220>  
<223> IRES-23

<400> 43  
gatccagagc aggaacagcg gaaacgagcg 30

<210> 44  
<211> 30  
<212> DNA  
<213> ARTIFICIAL

<220>  
<223> IRES-18

<400> 44  
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<210> 45  
<211> 30  
<212> DNA  
<213> ARTIFICIAL

<220>  
<223> IRES-16

<400> 45  
gatccaggaa ggtgaacgtc caatcgagcg 30

<210> 46  
 <211> 30  
 <212> DNA  
 <213> ARTIFICIAL

<220>  
 <223> IRES-15

<400> 46  
 gatccagagg agacgcatag agaacgagcg

30

<210> 47  
 <211> 42  
 <212> DNA  
 <213> ARTIFICIAL

<220>  
 <223> oligonucleotide containing 18 random nucleotide

<220>  
 <221> misc\_feature  
 <222> (13)..(30)  
 <223> n is either a, c, g, or t

<400> 47  
 acgcgtgacg cannnnnnnnn nnnnnnnnnn cgagcgacgc gt

42

<210> 48  
 <211> 135  
 <212> DNA  
 <213> ARTIFICIAL

<220>  
 <223> oligonucleotide containing two segments of 9 random nucleotides

<220>  
 <221> misc\_feature  
 <222> (69)..(77)  
 <223> n is either a, c, g, or t

<220>  
 <221> misc\_feature  
 <222> (87)..(95)  
 <223> n is either a, c, g, or t

<400> 48  
 ttaattaaga attcttctga cataaaaaaa aattctgaca taataaaaaa ttctgacata 60  
 aaaaaaaann nnnnnnnnaaa aaaaaannnn nnnnnnaaaaa aaaagactca caaccccaga 120  
 aacagacata cgcggt 135



<210> 49  
<211> 30  
<212> RNA  
<213> ARTIFICIAL  
  
<220>  
<223> ICS 1-23 a-b  
  
<400> 49  
gauccagagc aggaacagcg gaaacgagcg 30  
  
<210> 50  
<211> 15  
<212> RNA  
<213> ARTIFICIAL  
  
<220>  
<223> ICS 1-23 b  
  
<400> 50  
cagcggaac gagcg 15  
  
<210> 51  
<211> 14  
<212> RNA  
<213> *Saccharomyces cerevisiae*  
  
<400> 51  
uucucgauuc cgug 14  
  
<210> 52  
<211> 9  
<212> DNA  
<213> ARTIFICIAL  
  
<220>  
<223> 9 nt segment designated as ICA2-17.2  
  
<400> 52  
tccggtcgt 9  
  
<210> 53  
<211> 148  
<212> DNA  
<213> ARTIFICIAL  
  
<220>  
<223> two random 9 nt segments flanked by poly (a)<sub>9</sub> and embedded in bet  
a-globin 5' UT  
  
<400> 53  
gctagcttaa ttaagaattc ttctgacata aaaaaaatt ctgacataaa aaaaaattct 60

gacataaaaa aaaaggggtg tacaaaaaaa aaccagttt caaaaaaaaa gactcacaac 120  
cccagaaaca gacatacgcg tcgccatg 148

<210> 54  
<211> 148  
<212> DNA  
<213> ARTIFICIAL

<220>  
<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b  
eta-globin 5' UT

<400> 54  
gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattct 60  
gacataaaaa aaaaatgatt tgtaaaaaaa aagtgtcgat gaaaaaaaa gactcacaac 120  
cccagaaaca gacatacgcg tcgccatg 148

<210> 55  
<211> 147  
<212> DNA  
<213> ARTIFICIAL

<220>  
<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b  
eta-globin 5' UT

<400> 55  
gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaacacatcc cgaaaaaaaa attatatatc aaaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcgt cgccatg 147

<210> 56  
<211> 147  
<212> DNA  
<213> ARTIFICIAL

<220>  
<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b  
eta-globin 5' UT

<400> 56  
gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaaaccctgc tcaaaaaaaaa agcgtggtag aaaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcgt cgccatg 147

<210> 57  
<211> 147  
<212> DNA

<213> ARTIFICIAL

<220>

<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in beta-globin 5' UT

<400> 57

gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaaatccggg gtaaaaaaaa attaaataaa aaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcggt cgccatg 147

<210> 58

<211> 147

<212> DNA

<213> ARTIFICIAL

<220>

<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in beta-globin 5' UT

<400> 58

gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaaatccttaa agaaaaaaaa atgcagcgcg aaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcggt cgccatg 147

<210> 59

<211> 147

<212> DNA

<213> ARTIFICIAL

<220>

<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in beta-globin 5' UT

<400> 59

gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaatatcgct ttaaaaaaaaa aggttgcaact aaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcggt cgccatg 147

<210> 60

<211> 147

<212> DNA

<213> ARTIFICIAL

<220>

<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in beta-globin 5' UT

<400> 60

gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60

acataaaaaa aaattggttaa agaaaaaaaa agaagacccg aaaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcgt cgccatg 147

<210> 61  
<211> 147  
<212> DNA  
<213> ARTIFICIAL

<220>  
<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b  
eta-globin 5' UT

<400> 61  
gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaagcatgag taaaaaaaaa agctcaccta aaaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcgt cgccatg 147

<210> 62  
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<212> DNA  
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<220>  
<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b  
eta-globin 5' UT

<400> 62  
gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaatcggacg taaaaaaaaa atccggctgt aaaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcgt cgccatg 147

<210> 63  
<211> 147  
<212> DNA  
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<220>  
<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b  
eta-globin 5' UT

<400> 63  
gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaatttactg ctaaaaaaaa atggctgttc aaaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcgt cgccatg 147

<210> 64  
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<212> DNA  
<213> ARTIFICIAL

<220>  
<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b  
eta-globin 5' UT

<400> 64  
gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaattaagta gcaaaaaaaa attgtttagt aaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcgt cgccatg 147

<210> 65  
<211> 147  
<212> DNA  
<213> ARTIFICIAL

<220>  
<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b  
eta-globin 5' UT

<400> 65  
gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaaatcttgc gtaaaaaaaa agttcctgcg aaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcgt cgccatg 147

<210> 66  
<211> 147  
<212> DNA  
<213> ARTIFICIAL

<220>  
<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b  
eta-globin 5' UT

<400> 66  
gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaaagcatga gtaaaaaaaa aagctcacct aaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcgt cgccatg 147

<210> 67  
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<212> DNA  
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<220>  
<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b  
eta-globin 5' UT

<400> 67

21

gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaagcagtta ataaaaaaa agacatcagc aaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcggt cgccatg 147

<210> 68  
<211> 147  
<212> DNA  
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<220>  
<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b  
eta-globin 5' UT

<400> 68  
gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaaaaggctg ccaaaaaaaa aaacgttttag aaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcggt cgccatg 147

<210> 69  
<211> 147  
<212> DNA  
<213> ARTIFICIAL

<220>  
<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b  
eta-globin 5' UT

<400> 69  
gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaacttggtt caaaaaaaa aagttcctgt aaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcggt cgccatg 147

<210> 70  
<211> 147  
<212> DNA  
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<220>  
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eta-globin 5' UT

<400> 70  
gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaattcaaca tgaaaaaaa aagcgtctat aaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcggt cgccatg 147

<210> 71

<211> 147  
 <212> DNA  
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<220>  
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 eta-globin 5' UT

<400> 71  
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 ccagaaacag acatacgcgt cgccatg 147

<210> 72  
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<220>  
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 eta-globin 5' UT

<400> 72  
 gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
 acataaaaaa aaagcgcgtt agaaaaaaaa acctgccttc aaaaaaaaag actcacaacc 120  
 ccagaaacag acatacgcgt cgccatg 147

<210> 73  
 <211> 145  
 <212> DNA  
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<220>  
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 eta-globin 5' UT

<400> 73  
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 agaaacagac atacgcgtcg ccatg 145

<210> 74  
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 eta-globin 5' UT

<400> 74  
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acataaaaaa aaacgttgat tcaaaaaaaa aacgacctcc aaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcgt cgccatg 147

<210> 75  
<211> 146  
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<220>  
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eta-globin 5' UT

<400> 75  
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acataaaaaa aaagtcactg ccaaaaaaaa atcgatatta aaaaaaaga ctcacaaccc 120  
cagaaacaga catacgcgtc gccatg 146

<210> 76  
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<212> DNA  
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<220>  
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eta-globin 5' UT

<400> 76  
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acataaaaaa aaagagagtg agaaaaaaa atgtcctatt aaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcgt cgccatg 147

<210> 77  
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eta-globin 5' UT

<400> 77  
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acataaaaaa aaacgttgcg tcaaaaaaaa acgcccggcc aaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcgt cgccatg 147



<210> 78  
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 <212> DNA  
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 eta-globin 5' UT

<400> 78  
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 acataaaaaa aaacgcccggt gtaaaaaaaa acatggcggt aaaaaaaaag actcacaacc 120  
 ccagaaacag acatacgcggt cgccatg 147

<210> 79  
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 <212> DNA  
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<220>  
 <223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b  
 eta-globin 5' UT

<400> 79  
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 acataaaaaa aaacaatttc gtaaaaaaaa agtccgggac aaaaaaaaag actcacaacc 120  
 ccagaaacag acatacgcggt cgccatg 147

<210> 80  
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 <212> DNA  
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<220>  
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 eta-globin 5' UT

<400> 80  
 gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
 acataaaaaa aaaagatagc ctataaaaaa attgcttatg aaaaaaaaag actcacaacc 120  
 ccagaaacag acatacgcggt cgccatg 147

<210> 81  
 <211> 145  
 <212> DNA  
 <213> ARTIFICIAL

<220>  
 <223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b  
 eta-globin 5' UT

<400> 81  
gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaaccaggcg gaaaaaaaaa ctacttccaa aaaaaaagac tcacaacccc 120  
agaaacagac atacgcgctcg ccatg 145

<210> 82  
<211> 147  
<212> DNA  
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<220>  
<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b  
eta-globin 5' UT

<400> 82  
gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaataatcgt agaaaaaaaa aagcaggatg aaaaaaaaag actcacaacc 120  
ccagaaacag acatacgctg cgccatg 147

<210> 83  
<211> 147  
<212> DNA  
<213> ARTIFICIAL

<220>  
<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b  
eta-globin 5' UT

<400> 83  
gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaatggcatg agaaaaaaaa atgggcggct aaaaaaaaag actcacaacc 120  
ccagaaacag acatacgctg cgccatg 147

<210> 84  
<211> 146  
<212> DNA  
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<220>  
<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b  
eta-globin 5' UT

<400> 84  
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acataaaaaa aaattcaaca tgaaaaaaaa agcgtctata aaaaaaaga ctcacaaccc 120  
cagaaacaga catacgcgtc gccatg 146

<210> 85  
<211> 147  
<212> DNA  
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<220>

<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b  
eta-globin 5' UT

<400> 85  
gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaacggcgcc agaaaaaaaa aatgtgtggt aaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcgt cgccatg 147

<210> 86  
<211> 147  
<212> DNA  
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<220>

<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b  
eta-globin 5' UT

<400> 86  
gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaagtcatat caaaaaaaaa acgggtgagcg aaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcgt cgccatg 147

<210> 87  
<211> 147  
<212> DNA  
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<220>

<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b  
eta-globin 5' UT

<400> 87  
gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaagggcggcg gaaaaaaaaa atcgtttgcc aaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcgt cgccatg 147

<210> 88  
<211> 147  
<212> DNA  
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<220>

<223> two random 9 nt sequences flanked by poly (a) 9 and embedded in b

## eta-globin 5' UT

<400> 88  
gctagcttaa ttaagaattc ttctgacata aaaaaaaatt ctgacataaa aaaaaattcg 60  
acataaaaaa aaagtaaccc taaaaaaaaa aagcccgctt aaaaaaaaag actcacaacc 120  
ccagaaacag acatacgcggt cgccatg 147

<210> 89  
<211> 9  
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<220>  
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<400> 89  
gggttgtag 9

<210> 90  
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<400> 90  
cccagtttc 9

<210> 91  
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<400> 91  
atgatttgt 9

<210> 92  
<211> 9  
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<400> 92  
gtgtcgatg 9

<210> 93

<211> 9  
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<400> 93  
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<210> 94  
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<400> 94  
ttatatatc

9

<210> 95  
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<400> 95  
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9

<210> 96  
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<400> 96  
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9

<210> 97  
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<210> 98  
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<400> 98  
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<210> 99  
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<400> 99  
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<210> 100  
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<400> 100  
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<210> 101  
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<400> 101  
tatcgtctt

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<210> 102  
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<400> 102  
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<210> 103  
<211> 9  
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<400> 103  
ttggtaaag

9

<210> 104  
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<400> 104  
gaagaccg

9

<210> 105  
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<400> 105  
gcatgagta

9

<210> 106  
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<212> DNA  
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<400> 106  
gctcaccta

9

<210> 107  
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<400> 107  
tcggacgtt

9

<210> 108  
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<400> 108  
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9

<210> 109  
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<400> 109  
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9

<210> 110  
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9

<210> 111  
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9

<210> 112  
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<400> 112



ttgttttagt 9

<210> 113  
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<400> 113  
atcttgcg 9

<210> 114  
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<400> 114  
gttcctgcg 9

<210> 115  
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<400> 115  
agcatgagt 9

<210> 116  
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<400> 116  
agctcacct 9

<210> 117  
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<400> 117  
gcagttaat 9

<210> 118  
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gacatcagc 9

<210> 119  
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<400> 119  
aaggctgcc 9

<210> 120  
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<400> 120  
aacgttttag 9

<210> 121  
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<220>  
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<400> 121  
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<210> 122  
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<400> 122  
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<210> 123  
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<400> 124  
gcgtctat

8

<210> 125  
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<400> 125  
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9

<210> 126  
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<400> 126  
cccgttgt

9

<210> 127  
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9

<210> 128  
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9

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8

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gttcctgt

8

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<400> 131  
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9

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<400> 133  
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9

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<400> 134  
tcgatatt

8

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gagagtgg

9

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tgtcctatt

9

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9

<210> 138  
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9

<210> 139  
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cgcccgggt

9

<210> 140  
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<400> 140  
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<210> 141  
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<400> 141  
caatttcgt

9

<210> 142  
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<400> 142

gtccgggac

9

<210> 143

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<400> 143

agatagcct

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<210> 144

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<223> random 9 nt sequence

<400> 144

ttgcttatg

9

<210> 145

<211> 9

<212> DNA

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<223> random 9 nt sequence

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ccaggcgga

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<210> 146

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<400> 146

ctacttcc

8

<210> 147

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<400> 147  
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9

<210> 148  
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<400> 148  
agcaggatg

9

<210> 149  
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<400> 149  
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9

<210> 150  
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<400> 150  
tgggcggct

9

<210> 151  
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<400> 151  
ttcaacatg

9

<210> 152



<211> 8  
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<220>  
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gcgtctat

8

<210> 153  
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9

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9

<210> 155  
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<400> 155  
gtcatatca

9

<210> 156  
<211> 9  
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<400> 156  
cggtgagcg

9

41

<210> 157  
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<400> 157  
ggcggcgga

9

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<400> 158  
tcgtttgcc

9

<210> 159  
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<220>  
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<400> 159  
gtaacccta

9

<210> 160  
<211> 9  
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<220>  
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<400> 160  
agcccgctt

9

<210> 161  
<211> 156  
<212> DNA  
<213> Rattus norvegicus

<400> 161  
agtttcctcg gcggcgggag gcgagagcac taggagcaga gagagcgcgg gggccaccgg 60  
agacggcggc ggcggcgggc acgacgcgga cacagtcagg gtgcggcgga tcttcactt 120  
gcacacggag cagtcggtgg cccacgcagg atcacg 156

<210> 162  
 <211> 427  
 <212> DNA  
 <213> Rattus norvegicus

<400> 162  
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&lt;213&gt; Mus musculus

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 01/02586

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/11 C12N15/64 C12N15/67 C12N15/79 C12N15/85  
C12N5/10 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EMBASE, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	No relevant documents disclosed -----	

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*8\* document member of the same patent family

Date of the actual completion of the international search

28 June 2001

Date of mailing of the international search report

06/07/2001

Name and mailing address of the ISA

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## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-114

1). In view of the large number of claims (114 claims) at present on file and the large number of different oligonucleotide sequences claimed (194) and also the wording of the claims presently on file, which render it difficult, if not impossible, to determine the matter for which protection is sought, the present application fails to comply with the clarity and conciseness requirements of Article 6 PCT (see also Rule 6.1(a) PCT) to such an extent that a meaningful search is impossible.

2). Moreover, present claims relate to products and methods defined only by reference to a desirable characteristic or property, namely translational regulatory activity in a eukaryotic host cell selected from "(i) translational enhancing activity, (ii) translational inhibitory activity, (iii) internal ribosome entry site (IRES) activity, and (iv) any combination thereof". The claims cover all products and methods having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such products and methods. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the products and methods by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

3). In view of the reasons given under paragraphs (1) and (2) no search report has been established for the present application.

4). Furthermore, according to Rule 13 PCT an application must relate to one invention only or to a group of inventions so linked as to form a single general inventive concept, i.e. having at least one common technical defining a contribution over the known prior art. In the present case, the common technical feature among the different groups of inventions seems to be the fact that the claimed oligonucleotides comprise "a translational regulatory element comprising at least at least one oligonucleotide consisting of about 6-125 ribonucleotides, or a deoxyribonucleotide sequence encoding said oligonucleotide having translational regulatory activity in a eukaryotic cell". However, this single general concept lacks novelty and inventive activity with respect to the known prior art (see e.g. documents W099/61613, W098/37189, W094/23041, W098/54342, W000/44896, and W000/05366). Therefore 4 different main groups of inventions can be identified (see paragraph 2), which can be further subdivided into at least 194 different inventions comprising 194 different oligonucleotide sequences and a multiplicity of possible different combinations thereof.

5). Thus, the ISA fails to see which is the common inventive feature among the different groups of inventions defining an inventive contribution over the known prior art.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.